

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

27  
S/N: 09/872,086  
CHIR-15900/00US

(51) International Patent Classification <sup>5</sup> : <b>C12N 15/86, 7/01, A61K 39/12</b>		(11) International Publication Number: <b>WO 92/10578</b>
<b>A1</b>		(43) International Publication Date: <b>25 June 1992 (25.06.92)</b>
(21) International Application Number: <b>PCT/SE91/00855</b>		(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU*, TD (OAPI patent), TG (OAPI patent), US.
(22) International Filing Date: <b>12 December 1991 (12.12.91)</b>		
(30) Priority data: <b>9003978-5</b> <b>13 December 1990 (13.12.90)</b> <b>SE</b>		
(71) Applicant (for all designated States except US): <b>BIOPTION AB [SE/SE]; Eigentorpsvägen 16 ff, S-146 30 Tullinge (SE).</b>		
(72) Inventors; and (75) Inventors/Applicants (for US only): <b>GAROFF, Henrik [SE/SE]; Kroppåsbacken, S-126 58 Hägersten (SE). LILJESTRÖM, Peter [SE/SE]; Eigentorpsvägen 16, S-146 00 Tullinge (SE).</b>		
(74) Agent: <b>AB STOCKHOLMS PATENTBYRÅ; Zacco &amp; Bruhn, P.O. Box 23101, S-104 35 Stockholm (SE).</b>		Published With international search report.

(54) Title: **DNA EXPRESSION SYSTEMS BASED ON ALPHAVIRUSES**

## (57) Abstract

Efficient protein production from cloned DNA in animal cells has been hampered by the lack of suitable expression systems. The requirements of such an expression system are (1) to produce functional or immunogenic forms of protein molecules in a wide variety of animal cells, (2) high efficiency and (3) technical simplicity. The present invention is related to a technical solution to this problem. A DNA molecule encoding protein sequences is inserted into engineered variants of the cDNA of a positive stranded RNA virus genome from alphavirus which then, via RNA transcription and transfection into tissue culture cells, is used to produce either chimaeric virus particles for immunization or recombinant virus for protein production. Because of optimized conditions of transfection and the nature of the virus replication the present system combines both simplicity and safety in terms of handling, efficiency in terms of level of protein and RNA production, as well as broad host range.

# + DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

## FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU*	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

## DNA EXPRESSION SYSTEMS BASED ON ALPHAVIRUSES

The present invention is related to DNA expression systems based on alphaviruses, which systems can be used to transform animal cells for use in the production of desired products, such as proteins and vaccines, in high yields.

The rapid development of biotechnology is to a large extent due to the introduction of recombinant DNA technique, which has revolutionized cellbiological and medical research by opening new approaches to elucidate the molecular mechanisms of the cell. With the aid of the techniques of cDNA cloning, large numbers of interesting protein molecules are characterized each year. Therefore, a lot of research activity is today directed to elucidate the relationship between structure and function of these molecules. Eventually this knowledge will increase our possibilities to preserve healthiness and combat diseases in both humans and animals. Indeed, there is today a growing list of new "cloned" protein products that are already used as pharmaceuticals or diagnostics.

In the recombinant DNA approaches to study biological questions, DNA expression systems are crucial elements. Thus, efficient DNA expression systems, which are simple and safe to use, give high yields of the desired product and can be used in a variety of host cells, especially also in mammalian cells, are in great demand.

Many attempts have been made to develop DNA expression systems, which fulfill these requirements. Often, viruses have been used as a source of such systems. However, up to date none of the existing viral expression systems fulfill all these requirements in a satisfying way. For instance, the Baculovirus expression system for cDNA is extremely efficient but can be used only in insect cells (see Reference 1 of the list of cited references; for the sake of convenience, in the following the cited references are only identified by the number they have on said list). As many important molecules will have to be produced and processed in

cells of mammalian origin in order for them to become active, this system cannot be used in such cases. Furthermore, the Baculovirus cDNA expression system is not practically convenient for analysis of the relationship between structure and function of a protein because this involves in general the analysis of whole series of mutant variants. Today it takes about 6-8 weeks to construct a single Baculo recombinant virus for phenotype analyses. This latter problem is also true for the rather efficient Vaccinia recombinant virus and other contemporary recombinant virus cDNA expression systems (2,3). The procedure to establish stably transformed cell lines is also a very laborious procedure, and in addition, often combined with very low levels of protein expression.

Hitherto, most attempts to develop viral DNA expression systems have been based on viruses having DNA genomes or retroviruses, the replicative intermediate of the latter being double stranded DNA.

Recently, however, also viruses comprising RNA genomes have been used to develop DNA expression systems.

In EP 0 194 809 RNA transformation vectors derived from (+) strand RNA viruses are disclosed which comprise capped viral RNA that has been modified by insertion of exogenous RNA into a region non-essential for replication of said virus RNA genome. These vectors are used for expression of the function of said exogenous RNA in cells transformed therewith. The RNA can be used in solution or packaged into capsids. Furthermore, this RNA can be used to generate new cells having new functions, i.e. protein expression. The invention of said reference is generally claimed as regards host cells, (+) strand RNA viruses and the like. Nevertheless, it is obvious from the experimental support provided therein that only plant cells have been transformed and in addition only Bromo Mosaic virus, a plant

virus, has been used as transformation vector.

Although it is stated in said reference that it is readily apparent to those skilled in the art to convert any RNA virus-cell system to a useful expression system for exogenous DNA using principals described in the reference, this has not been proven to be true in at least the case of animal cell RNA viruses. The reasons for this seem to be several. These include:

- 1) Inefficiencies in transfecting animal cells with in vitro transcribed RNA;
- 2) Inefficiency of apparently replication competent RNA transcripts to start RNA replication after commonly used transfection procedures;
- 3) The inability to produce high titre stocks of recombinant virus that does not contain any helper virus;
- 4) The inability to establish stable traits of transformed cells expressing the function of the exogenous RNA.

In Proc. Natl. Acad. Sci. USA, Vol 84, 1987, pp 4811-4815 a gene expression system based on a member of the Alphavirus genus, viz. Sindbis virus, is disclosed which is used to express the bacterial CAT (chloramphenicol acetyltransferase) gene in avian cells, such as chicken embryo fibroblasts.

Xiong et al., Science, Vol 243, 1989, 1188-1191 also disclose a gene expression system based on Sindbis virus. This system is said to be efficient in a broad range of animal cells. Expression of the bacterial CAT gene in insect, avian and mammalian cells inclusive of human cells is disclosed therein.

Even though it is known from prior art that one

member of the Alphavirus genus, the Sindbis virus, can tolerate insertion and direct the expression of at least one foreign gene, the bacterial chloramphenicol acetyl transferase (CAT) gene, it is evident from the results described that both systems described above are both ineffective in terms of exogenous gene expression and also very cumbersome to use. Hence, neither system has found any usage in the field of DNA expression in animal cells today.

In the first example a cDNA copy of a defective interfering (DI) virus variant of Sindbis virus was used to carry the CAT gene. RNA was transcribed in vitro and used to transfect avian cells and some CAT protein production could be demonstrated after infecting cells with wild-type Sindbis virus. The latter virus provided the viral replicase for expression of the CAT construct. The inefficiency of this system depends on 1) low level of initial DI-CAT RNA transfection (0.05-0.5 % of cells) and 2) inefficient usage of the DI-CAT RNA for protein translation because of unnatural and suboptimal protein initiation translation signals. This same system also results in packaging of some of the recombinant DI-CAT genomes into virus particles. However, this occurs simultaneously with a very large excess of wild-type Sindbis virus production. Therefore, the usage of this mixed virus stock for CAT expression will be much hampered by the fact that most of the replication and translation activity of the cells infected with such a stock will deal with the wild-type and not with recombinant gene expression.

Much of the same problems are inherent to the other Sindbis expression system described. In this an RNA replication competent Sindbis DNA vector is used to carry the CAT gene. RNA produced in vitro is shown to replicate in animal cells and CAT activity is found. However, as only a very low number of cells are transfected the overall CAT production remains low. Another

possible explanation for this is that the Sindbis construct used is not optimal for replication. Wild-type Sindbis virus can be used to rescue the recombinant genome into particles together with an excess of wild-type genomes and this mixed stock can then be used to express a CAT protein via infection. However, this stock has the same problems as described above for the recombinant DI system. The latter paper shows also that if virus is amplified by several passages increased titres of the recombinant virus particles can be obtained. However, one should remember that the titre of the wild-type virus will increase correspondingly and the original problem of mostly wild-type virus production remains. There are also several potential problems when using several passages to produce a mixed virus stock. As there is no selected pressure for preservation of the recombinant genomes these might easily 1) undergo rearrangements and 2) become outnumbered by wild-type genomes as a consequence of less efficient replication and/or packaging properties.

Another important aspect of viral DNA expression vectors is use thereof to express antigens of unrelated pathogens and thus they can be used as vaccines against such pathogens.

Development of safe and effective vaccines against viral diseases has proven to be quite a difficult task. Although many existing vaccines have helped to combat the worldwide spread of many infectious diseases, there is still a large number of infectious agents against which effective vaccines are missing. The current procedures of preparing vaccines present several problems: (1) it is often difficult to prepare sufficiently large amounts of antigenic material; (2) In many cases there is the additional hazard that the vaccine preparation is not killed or sufficiently attenuated; (3) Effective vaccines are often hard to produce since there is a major difficulty in presenting the antigenic epitope in



an immunologically active form; (4) In the case of many viruses, genetic variations in the antigenic components results in the evolution of new strains with new serological specificities, which again creates a need for the development of new vaccines.

Two types of viral DNA vectors have been developed in order to overcome many of these problems in vaccine production. These either provide recombinant viruses or provide chimaeric viruses. The recombinant viruses contain a wild-type virus package around a recombinant genome. These particles can be used to infect cells which then produce the antigenic protein from the recombinant genome. The chimaeric viruses also contain a recombinant genome but this specifies the production of an antigen, usually as part of a normal virus structural protein, which then will be packaged in progeny particles and e.g. exposed on the surface of the viral spike proteins. The major advantages of these kind of virus preparations for the purpose of being used as a vaccine are 1) that they can be produced in large scale and 2) that they provide antigen in a natural form to the immunological system of the organism. Cells, which have been infected with recombinant viruses, will synthesize the exogenous antigen product, process it into peptides that then present them to T cells in the normal way. In the case of the chimaeric virus there is, in addition, an exposition of the antigen in the context of the subunits of the virus particle itself. Therefore, the chimaeric virus is also called an epitope carrier.

The major difficulty with these kind of vaccine preparations are, how to ensure a safe and limited replication of the particles in the host without side effects. So far, some success has been obtained with vaccinia virus as an example of the recombinant virus approach (69) and of polio virus as an example of a chimaeric particle (70-72). As both virus variants are

based on commonly used vaccine strains one might argue that they could be useful vaccine candidates also as recombinant respectively chimaeric particles (69-72). However, both virus vaccines are combined with the risk for side effects, even severe ones, and in addition these virus strains have already been used as vaccines in large parts of the population in many countries.

As is clear from the afore mentioned discussion there is much need to develop improved DNA expression systems both for an easy production of important proteins or polypeptides in high yields in various kinds of animal cells and for the production of recombinant viruses or chimaeric viruses to be used as safe and efficient vaccines against various pathogenes.

Thus, an object of the present invention is to provide an improved DNA expression system based on virus vectors which can be used both to produce proteins and polypeptides and as recombinant virus or chimaeric virus, which system offers many advantages over prior art.

To that end, according to the present invention there is provided an RNA molecule derived from an alphavirus RNA genome and capable of efficient infection of animal host cells, which RNA molecule comprises the complete alphavirus RNA genome regions, which are essential to replication of the said alphavirus RNA, and further comprises an exogenous RNA sequence capable of expressing its function in said host cell, said exogenous RNA sequence being inserted into a region of the RNA molecule which is non-essential to replication thereof.

Alphavirus is a genus belonging to the family Togaviridae having single stranded RNA genomes of positive polarity enclosed in a nucleocapsid surrounded by an envelope containing viral spike proteins.

The Alphavirus genus comprises among others the Sindbis virus, the Semliki Forest virus (SFV) and the Ross River virus, which are all closely related.

According to a preferred embodiment of the invention, the Semliki Forest virus (SFV) is used as the basis of the DNA expression system.

5 The exogenous RNA sequence encodes a desired genetic trait, which is to be conferred on the virus or the host cell, and said sequence is usually complementary to a DNA or cDNA sequence encoding said genetic trait. Said DNA sequence may be comprised of an isolated  
10 natural gene, such as a bacterial or mammalian gene, or may constitute a synthetic DNA sequence coding for the desired genetic trait i.e. expression of a desired product, such as an enzyme, hormone, etc. or expression of a peptide sequence defining an exogenous antigenic epitope or determinant.

15 If the exogenous RNA sequence codes for a product, such as a protein or polypeptide, it is inserted into the viral RNA genome replacing deleted structural protein encoding region(s) thereof, whereas a viral epitope encoding RNA sequence may be inserted into  
20 structural protein encoding regions of the viral RNA genome, which essentially do not comprise deletions or only have a few nucleosides deleted.

The RNA molecule can be used per se, e.g. in solution to transform animal cells by conventional transfection,  
25 e.g. the DEAE-Dextran method or the calcium phosphate precipitation method. However, the rate of transformation of cells, and, thus the expression rate can be expected to increase substantially if the cells are transformed by infection with infectious viral particles. Thus, a suitable embodiment of the invention is  
30 related to an RNA virus expression vector comprising the RNA molecule of this invention packaged into infectious particles comprising the said RNA within the alphavirus nucleocapsid and surrounded by the membrane including the alphavirus spike proteins.

35 The RNA molecule of the present invention can be packaged into such particles without restraints pro-

vided that it has a total size corresponding to the wild type alphavirus RNA genome or deviating therefrom to an extent compatible with package of the said RNA into the said infectious particles.

5        These infectious particles, which include recombinant genomes packaged to produce a pure, high titre recombinant virus stock, provides a means for exogenous genes or DNA sequences to be expressed by normal virus particle infection, which as regards transformation  
10        degree, is much more efficient than RNA transfection.

      According to a suitable embodiment of the invention such infectious particles are produced by cotransfection of animal host cells with the present RNA which  
15        lacks part of or the complete region(s) encoding the structural viral proteins together with a helper RNA molecule transcribed in vitro from a helper DNA vector comprising the SP6 promoter region, those 5' and 3' regions of the alphavirus cDNA which encode cis acting  
20        signals needed for RNA replication and the region encoding the viral structural proteins but lacking essentially all of the nonstructural virus proteins encoding regions including sequences encoding RNA signals for packaging of RNA into nucleocapsid particles, and culturing the host cells.

25        According to another aspect of the invention efficient introduction of the present RNA into animal host cells can be achieved by electroporation. For example, in the case of Baby Hamster Kidney (BHK) cells a transformation degree of almost 100 % has been obtained for  
30        the introduction of an RNA transcript derived from SFV cDNA of the present invention. This makes it possible to reach so high levels of exogenous protein production in every cell that the proteins can be followed in total cell lysates without the need of prior concentration by antibody precipitation.  
35       

      By electroporation, it is also possible to obtain a high degree of cotransfection in the above process for

production of infectious particles comprising packaged RNA of the present invention. Essentially all animal cells will contain both the present RNA molecule and the helper RNA molecule, which leads to a very efficient trans complementation and formation of infectious  
5 particles. A pure recombinant virus stock, consisting of up to  $10^9$ - $10^{10}$  infectious particles, can be obtained from  $5 \times 10^6$  cotransfected cells after only a 24 h incubation. Furthermore, the so obtained virus stock is  
10 very safe to use, since it is comprised of viruses containing only the desired recombinant genome, which can infect host cells but can not produce new progeny virus.

Theoretically, a regeneration of a wild-type virus  
15 genome could take place when producing the recombinant virus in the contransfected cells. However, the possibility to avoid spread of such virus can be eliminated by incorporating a conditionally lethal mutation into the structural part of the helper genome. Such a muta-  
20 tion is described in the experimental part of this application. Thus, the virus produced with such a helper will be noninfectious if not treated in vitro under special conditions.

The technique of electroporation is well known within  
25 the field of biotechnology and optimal conditions can be established by the man skilled in the art. For instance, a BioRad Gene pulser apparatus (BioRad, Richmond, CA, USA) can be used to perform said process.

The RNA molecule of the present invention is derived  
30 by in vivo or in vitro transcription of a cDNA clone, originally produced from an alphavirus RNA and comprising an inserted exogenous DNA fragment encoding a desired genetic trait.

Accordingly, the present invention is also related to  
35 a DNA expression vector comprising a full-length or partial cDNA complementary to alphavirus RNA or parts thereof and located immediately downstream of the SP6

RNA polymerase promoter and having a 5'ATGG, a 5'GATGG or any other 5' terminus and a TTTCCA<sub>69</sub>ACTAGT or any other 3' terminus.

5 According to one aspect of the present invention portions of the viral cDNA are deleted, the deletions comprising the complete or part of the region(s) encoding the virus structural proteins, and the vector further comprises an integrated polylinker region, which may correspond to BamHI-SmaI-XmaI, inserted at a  
10 location which enables an exogenous DNA fragment encoding a foreign polypeptide or protein to be inserted into the vector cDNA for subsequent expression in an animal host cell.

15 According to another aspect of this invention, the vector is comprised of full-length cDNA wherein an exogenous DNA fragment encoding a foreign epitopic peptide sequence can be inserted into a region coding for the viral structural proteins.

20 It is appreciated that this cDNA clone with its exogenous DNA insert is very efficiently replicated after having been introduced into animal cells by transfection.

A very important aspect of the present invention is that it is applicable to a broad range of host cells of  
25 animal origin. These host cells can be selected from avian, mammalian, reptilian, amphibian, insect and fish cells. Illustrative of mammalian cells are human, monkey, hamster, mouse and porcine cells. Suitable avian cells are chicken cells, and as reptilian cells  
30 viper cells can be used. Cells from frogs and from mosquitoes and flies (*Drosophila*) are illustrative of amphibian and insecticidal cells, respectively. A very efficient virus vector/host cell system according to the invention is based on SFV/BHK cells, which will be  
35 discussed more in detail further below.

However, even though a very important advantage of the present DNA expression vector is that it is very

efficient in a broad variety of animal cells it can also be used in other eucaryotic cells and in pro-caryotic cells.

5       The present invention is also related to a method to produce transformed animal host cells comprising transfection of the cells with the present RNA molecule or with the present transcription vector comprised of cDNA and carrying an exogenous DNA fragment. According to a suitable embodiment of the invention, transfection is produced by the above mentioned electroporation method, 10 a very high transfection rate being obtained.

15       A further suitable transformation process is based on infection of the animal host cells with the above mentioned infectious viral particles comprising the present RNA molecule.

      The transformed cells of the present invention can be used for different purposes.

20       One important aspect of the invention is related to use of the present transformed cells to produce a polypeptide or a protein by culturing the transformed cells to express the exogenous RNA and subsequent isolation and purification of the product formed by said expression. The transformed cells can be produced by infection with the present viral particles comprising exogenous RNA encoding the polypeptide or protein as mentioned above, or by transfection with an RNA transcript 25 obtained by in vitro transcription of the present DNA vector comprised of cDNA and carrying an exogenous DNA fragment coding for the polypeptide or the protein.

30       Another important aspect of the invention is related to use of the present transformed cells for the production of antigens comprised of chimaeric virus particles for use as immunizing component in vaccines or for immunization purposes for in vivo production of immunizing components for antisera production. 35

      Accordingly, the present invention is also related to an antigen consisting of a chimaeric alphavirus having

an exogenous epitopic peptide sequence inserted into its structural proteins.

Preferably, the chimaeric alphavirus is derived from SFV.

5 According to a suitable embodiment, the exogenous epitopic peptide sequence is comprised of an epitopic peptide sequence derived from a structural protein of a virus belonging to the immunodeficiency virus class inclusive of the human immunodeficiency virus types.

10 A further aspect of the invention is related to a vaccine preparation comprising the said antigen as immunizing component.

15 In said vaccine the chimaeric alphavirus is suitably attenuated by comprising mutations, such as the conditionally lethal SFV-mutation described before, amber (stop codon) or temperature sensitive mutations, in its genome.

20 For instance, if the chimaeric virus particles containing the afore mentioned conditional lethal mutation in its structural proteins (a defect to undergo a certain proteolytical cleavage in host cell during morphogenesis) is used as a vaccine then this is first activated by limited proteolytic treatment before given to the organism so that it can infect recipient cells.  
25 New chimaeric particles will be formed in cells infected with the activated virus but these will again be of the lethal phenotype and further spread of infection is not possible.

30 The invention is also concerned with a method for the production of the present antigen comprising

a) in vitro transcription of the cDNA of the present DNA vector carrying an exogenous DNA fragment encoding the foreign epitopic peptide sequence and transfection of animal host cells with the produced RNA transcript,  
35 or

b) transfection of animal host cells with the said cDNA of the above step a),



culturing the transfected cells and recovering the chimaeric alphavirus antigen. Preferably, transfection is produced by electroporation.

5 Still another aspect of the invention is to use a recombinant virus containing exogenous RNA encoding a polypeptide antigen for vaccination purpose or to produce antisera. In this case the recombinant virus or the conditionally lethal variant of it is used to infect cells in vivo and antigen production will take place in the infectious cells and used for antigen presentation to the immunological system.

10 According to another embodiment of the invention, the present antigen is produced in an organism by using in vivo infection with the present infectious particles containing exogenous RNA encoding an exogenous epitopic peptide sequence.

15 In the following, the present invention will be illustrated more in detail with reference to the Semliki Forest virus (SFV), which is representative for the alphaviruses. This description can be more fully understood in conjunction with the accompanying drawings in which:

20 Fig. 1 is a schematic view over the main assembly and disassembly events involved in the life cycle of the Semliki Forest virus, and also shows regulation of the activation of SFV entry functions by p62 cleavage and pH;

25 Fig. 2 illustrates the use of translocation signals during synthesis of the structural proteins of SFV; top, the gene map of the 26S subgenomic RNA; middle, the process of membrane translocation of the p62, 6K and E1 proteins; small arrows on the luminal side denote signal peptidase cleavages; at the bottom, the characteristics of the three signal peptides are listed;

30 Fig. 3 shows features that make SFV an excellent

choice as an expression vector;

5 Fig. 4 A-C show the construction of full-length infectious clones of SFV; Fig. 4A shows a schematic restriction map of the SFV genome; primers used for initiating cDNA synthesis are indicated as arrows, and the cDNA inserts used to assemble the final clone are showed as bars; Fig. 4B shows plasmid pPLH211, i.e. the SP6 expression vector used as carrier for the full-length infectious clone of SFV, and the resulting 10 plasmid pSP6-SFV4; Fig. 4C shows the structure of the SP6 promoter area of the SFV clone; the stippled bars indicate the SP6 promoter sequence, and the first nucleotide to be transcribed is marked by an asterisk; underlined regions denote authentic SFV sequences;

15 Fig. 5 shows the complete nucleotide sequence of the pSP6-SFV4 RNA transcript as DNA (U = T) and underneath the DNA sequence, the amino acid sequence of the non-structural polyprotein and the structural polyprotein;

20 Fig. 6 shows an SFV cDNA expression system for the production of virus after transfection of in vitro made RNA into cells;

Fig. 7 shows the construction of the SFV expression vectors pSFV1-3 and of the Helper 1;

25 Fig. 8 shows the polylinker region of SFV vector plasmids pSFV1-3; the position of the promoter for the subgenomic 26S RNA is boxed, and the first nucleotide to be transcribed is indicated by an asterisk;

30 Fig. 9 is a schematic presentation of in vivo packaging of pSFV1-dhfr RNA into infectious particles using helper trans complementation; (dhfr means dihydrofolate reductase)

Fig. 10 shows the use of trypsin to convert p62-containing noninfectious virus particles to infectious particles by cleavage of p62 to E2 and E3;

35 Fig. 11 shows the expression of heterologous proteins in BHK cells upon RNA transfection by electroporation; and

Fig. 12 shows in its upper part sequences encompassing the major antigenic site of SFV and the in vitro made substitutions leading to a BamHI restriction endonuclease site, sequences spanning the principal neutralizing domain of the HIV gp120 protein, and the HIV domain inserted into the SFV carrier protein E2 as a BamHI oligonucleotide; and its lower part is a schematic presentation of the SFV spike structure with blow-ups of domain 246-251 in either wild type or chimaeric form.

The alphavirus Semliki Forest virus (abbreviated SFV in the following text) has for some 20 years been used as model system in both virology and cell biology to study membrane biosynthesis, membrane structure and membrane function as well as protein-RNA interactions (4, 5). The major reason for the use of SFV as such a model is due to its simple structure and efficient replication.

With reference to Fig. 1-3, in the following the SFV and its replication are explained more in detail. In essential parts, this disclosure is true also for the other alphaviruses, such as the Sindbis virus, and many of the references cited in this connection are indeed directed to the Sindbis virus. SFV consists of an RNA-containing nucleocapsid and a surrounding membrane composed of a lipid bilayer and proteins, a regularly arranged icosahedral shell of a protein called C protein forming the capsid inside which the genomic RNA is packaged. The capsid is surrounded by the lipid bilayer that contains three proteins called E1, E2, and E3. These so-called envelope proteins are glycoproteins and their glycosylated portions are on the outside of the lipid bilayer, complexes of these proteins forming the "spikes" that can be seen in electron micrographs to project outward from the surface of the virus.

The SFV genome is a single-stranded 5'-capped and 3'-polyadenylated RNA molecule of 11422 nucleotides (6,7).

It has positive polarity, i.e. it functions as an mRNA, and naked RNA is able to start an infection when introduced into the cytoplasm of a cell. Infection is initiated when the virus binds to protein receptors on the host cell plasma membrane, whereby the virions become selectively incorporated into "coated pits" on the surface of the plasma membrane, which invaginate to form coated vesicles inside the cell, whereafter said vesicles bearing endocytosed virions rapidly fuse with organelles called endosomes. From the endosome, the virus escapes into the cell cytosol as the bare nucleocapsid, the viral envelope remaining in the endosome. Thereafter, the nucleocapsid is "uncoated" and, thus, the genomic RNA is released. Referring now to Fig. 1, infection then proceeds with the translation of the 5' two-thirds of the genome into a polyprotein which by self-cleavage is processed to the four nonstructural proteins nsP1-4 (8). Protein nsP1 encodes a methyl transferase which is responsible for virus-specific capping activity as well as initiation of minus strand synthesis (9, 10); nsP2 is the protease that cleaves the polyprotein into its four subcomponents (11, 12); nsP3 is a phosphoprotein (13, 14) of as yet unknown function, and nsP4 contains the SFV RNA polymerase activity (15, 16). Once the nsP proteins have been synthesized they are responsible for the replication of the plus strand (42S) genome into full-length minus strands. These molecules then serve as templates for the production of new 42S genomic RNAs. They also serve as templates for the synthesis of subgenomic (26S) RNA. This 4073 nucleotides long RNA is colinear with the last one-third of the genome, and its synthesis is internally initiated at the 26S promoter on the 42S minus strands (17, 18).

The capsid and envelope proteins are synthesized in different compartments, and they follow separate pathways through the cytoplasm, viz. the envelope proteins

are synthesized by membrane-bound ribosomes attached to the rough endoplasmic reticulum, and the capsid protein is synthesized by free ribosomes in the cytosol. However, the 26S RNA codes for all the structural proteins of the virus, and these are synthesized as a poly-protein precursor in the order C-E3-E2-6K-E1 (19). Once the capsid (C) protein has been synthesized it folds to act as a protease cleaving itself off the nascent chain (20, 21). The synthesized C proteins bind to the recently replicated genomic RNA to form new nucleocapsid structures in the cell cytoplasm.

The said cleavage reveals an N-terminal signal sequence in the nascent chain which is recognized by the signal recognition particle targeting the nascent chain - ribosome complex to the endoplasmic reticulum (ER) membrane (22, 23), where it is cotranslationally translocated and cleaved by signal peptidase to the three structural membrane proteins p62 (precursor form of E3/E2), 6K and E1 (24, 25). The translocational signals used during the synthesis of the structural proteins are illustrated in Fig. 2. The membrane proteins undergo extensive posttranslational modifications within the biosynthetic transport pathway of the cell. The p62 protein forms a heterodimer with E1 via its E3 domain in the endoplasmic reticulum (26). This dimer is transported out to the plasma membrane, where virus budding occurs through spike nucleocapsid interactions. At a very late (post-Golgi) stage of transport the p62 protein is cleaved to E3 and E2 (27), the forms that are found in mature virions. This cleavage activates the host cell binding function of the virion as well as the membrane fusion potential of E1. The latter activity is expressed by a second, low-pH activation step after the virus enters the endosomes of a new host cell and is responsible for the release of the viral nucleocapsid into the cell cytoplasm (28-32). The mature virus particles contain one single copy of the RNA

genome encapsidated within 180 copies of the capsid protein in a T=3 symmetry, and is surrounded by a lipid bilayer carrying 240 copies of the spike trimer protein consisting of E1+E2+E3 arranged in groups of three in a T=4 symmetry (33).

The SFV entry functions are activated and regulated by p62 cleavage and pH. More specifically, the p62-E1 heterodimers formed in the ER are acid resistant. When these heterodimers are transported to the plasma membrane via the Golgi complex the E1 fusogen cannot be activated in spite of the mildly acidic environment, since activation requires dissociation of the complex. As is illustrated in Fig. 1, the released virus particles contain E2E1 complexes. Since the association between E2 and E1 is sensitive to acidic pH, during entry of the virus into a host cell through endocytosis the acidic milieu of the endosome triggers the dissociation of the spike complex (E1 E2 E3) resulting in free E1. The latter can be activated for the catalysis of the fusion process between the viral and endosomal membranes in the infection process as disclosed above.

As indicated in the preceding parts of the disclosure, the alphavirus system, and especially the SFV system, has several unique features which are to advantage in DNA expression systems. These are summarized below with reference to Fig. 3.

1. Genome of positive polarity. The SFV RNA genome is of positive polarity, i.e. it functions directly as mRNA, and infectious RNA molecules can thus be obtained by transcription from a full-length cDNA copy of the genome.

2. Efficient replication. The infecting RNA molecule codes for its own RNA replicase, which in turn drives an efficient RNA replication. Indeed, SFV is one of the most efficiently replicating viruses known. Within a few hours up to 200.000 copies of the plus-RNAs are made in a single cell. Because of the abundance of

great us to uphold safety when massproduction of virus stocks is necessary.

In the nucleotide and amino acid sequences the following abbreviations have been used in this specification:

Ala, alanine; Ile, isoleucine; Leu, leucine; Met, methionine; Phe, phenylalanine; Pro, proline; Trp, tryptophan; Val, valine; Asn, asparagine; Cys, cysteine; Gln, glutamine; Gly, glycine; Ser, serine; Thr, threonine; Tys, tyrosine; Arg, arginine; His, histidine; Lys, lysine; Asp, aspartic acid; Glu, glutamic acid; A, adenine; C, cytosine; G, guanine; T, thymine; U, uracil.

The materials and the general methodology used in the following examples are disclosed below.

1. Materials. Most restriction enzymes, DNA Polymerase I, Klenow fragment, calf intestinal phosphatase, T4 DNA ligase and T4 Polynucleotide kinase were from Boehringer (Mannheim, FRG). SphI, StuI and KpnI together with RNase inhibitor (RNasin) and SP6 Polymerase were from Promega Biotec (Madison, WI). Sequenase (Modified T7 polymerase) was from United States Biochemical (Cleveland, Ohio). Proteinase K was from Merck (Darmstadt, FRG). Ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides and the cap analogue m<sup>7</sup>G(5')ppp(5')G were from Pharmacia (Sweden). Oligonucleotides were produced using an Applied Biosystems synthesizer 380B followed by HPLC and NAP-5 (Pharmacia) purification. Spermidine, phenylmethylsulfonyl fluoride (PMSF), diethylpyrocarbonate (DEPC), bovine serum albumin (BSA), creatine phosphate and creatine phosphokinase were from Sigma (St. Louis, Mo). Pansorbin was from CalBiochem (La Jolla, CA). Agarose was purchased from FMC BioProducts (Rockland, Maine), and acrylamide from BioRad (Richmond, CA). L-[<sup>35</sup>S]-methionine and α-[<sup>35</sup>S]-dATP-α-S were from Amersham.

2. Virus growth and purification: BHK-21 cells were

these molecules practically all ribosomes of the infected cell will be enrolled in the synthesis of the virus encoded proteins, thus overtaking host protein synthesis (34), and pulse-labelling of infected cells results in almost exclusive labelling of viral proteins. During a normal infection  $10^5$  new virus particles are produced from one single cell, which calculates to at least  $10^8$  protein molecules encoded by the viral genome (5).

3. Cytoplasmic replication. SFV replication occurs in the cell cytoplasm, where the virus replicase transcribes and caps the subgenomes for production of the structural proteins (19). It would obviously be very valuable to include this feature in a cDNA expression system to eliminate the many problems that are encountered in the conventional "nuclear" DNA expression systems, such as mRNA splicing, limitations in transcription factors, problems with capping efficiency and mRNA transport.

4. Late onset of cytopathic effects. The cytopathic effects in the infected cells appear rather late during infection. Thus, there is an extensive time window from about 4 hours after infection to up to 24 hours after infection during which a very high expression level of the structural proteins is combined with negligible morphological change.

5. Broad host range. This phenomenon is probably a consequence of the normal life cycle which includes transmission through arthropod vectors to wild rodents and birds in nature. Under laboratory conditions, SFV infects cultured mammalian, avian, reptilian and insect cells (35) (Xiong, et al, loc. cit.)

6. In nature SFV is of very low pathogenicity for humans. In addition, the stock virus produced in tissue culture cells is apparently apathogenic. By means of specific mutations it is possible to create conditionally lethal mutations of SFV, a feature that is of



grown in BHK medium (Gibco Life Technologies, Inc., New York) supplemented with 5 % fetal calf serum, 10 % tryptose phosphate broth, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 2 mM glutamine. 90 % confluent monolayers were washed once with PBS and infected with SFV in MEM containing 0.2 % bovine serum albumin (BSA), 10 mM HEPES and 2 mM glutamine at a multiplicity of 0.1. Twenty-four hours post infection (p.i.) the medium was collected and cell debris removed by centrifugation at 8,000 xg for 20 min at 4°C. The virus was pelleted from the medium by centrifugation at 26,000 rpm for 1.5 h in an SW28 rotor at 4°C. The virus was resuspended in TN containing 0.5 mM EDTA.

3. Metabolic labeling and immunoprecipitation. Confluent monolayers of BHK cells grown in MEM supplemented with 10 mM HEPES, 2 mM glutamine, 0.2 % BSA, 100 IU/mol of penicillin and 100 µg/ml streptomycin, were infected at a multiplicity of 50 at 37°C. After 1 h p.i. the medium was replaced with fresh and growth continued for 3.5 h. The medium was removed and cells washed once with PBS and overlaid with methionine-free MEM containing 10 mM HEPES and 2 mM glutamine. After 30 min at 37°C the medium was replaced with the same containing 100 µCi/ml of [<sup>35</sup>S]methionine (Amersham) and the plates incubated for 10 min at 37°C. The cells were washed twice with labeling medium containing 10X excess methionine and then incubated in same medium for various times. The plates were put on ice, cells washed once with ice-cold PBS and finally lysis buffer (1 % NP-40 - 50 mM Tris-HCl, pH 7.6 - 150 mM NaCl - 2 mM EDTA) containing 10 µg/ml PMSF (phenylmethylsulfonyl fluoride) was added. Cells were scraped off the plates, and nuclei removed by centrifugation at 6,000 rpm for 5 min at 4°C in an Eppendorf centrifuge. Immunoprecipitations of proteins was performed as described (31). Briefly, antibody was added to lysate and the mixture

kept on ice for 30 min. Complexes were recovered by binding to Pansorbin for 30 min on ice. Complexes were washed once with low salt buffer, once with high salt buffer, and once with 10 mM Tris-HCl, pH 7.5, before heating with gel loading buffer. To precipitate dhfr, SDS was added to 0.1 % and the mixture heated to 95°C for 2 min followed by addition of 10 volumes of lysis buffer. Anti-E1 [8.139], anti-E2 [5.1] (36), and anti-C [12/2] (37) monoclonals have been described. The human transferrin receptor was precipitated with the monoclonal antibody OKT-9 in ascites fluid. This preparation was provided by Thomas Ebel at our laboratory using a corresponding hybridoma cell line obtained from ATCC (American Typ Culture Collection) No CRL 8021. Polyclonal rabbit anti-mouse dhfr was a kind gift from E. Hurt (European Molecular Biology Laboratory, Heidelberg, FRG) and rabbit anti-lysozyme has been described (38).

4. Immunofluorescence. To perform indirect immunofluorescence, infected cell monolayers on glass coverslips were rinsed twice with phosphate-buffered saline (PBS) and fixed in -20°C methanol for 6 min. After fixation, the methanol was removed and the coverslip washed 3 times with PBS. Unspecific antibody binding was blocked by incubation at room temperature with PBS containing 0.5 % gelatin and 0.25 % BSA. The blocking buffer was removed and replaced with same buffer containing primary antibody. After 30 min at room temperature the reaction was stopped by washing 3 times with PBS. Binding of secondary antibody (FITC-conjugated sheep anti-mouse [BioSys, Compiègne, France]) was done as for the primary antibody. After 3 washes with PBS and one rinse with water the coverslip was allowed to dry before mounting in Moviol 4-88 (Hoechst, Frankfurt am Main, FRG) containing 2.5 % DABCO (1,4-diazobicyclo-[2.2.2]-octane).

5. DNA procedures. Plasmids were grown in Escherichia

coli DH5 $\alpha$  (Bethesda Research Laboratories) [recA endA1  
gyrA96 thi1 hsdR17 supE44 relA1  $\Delta$ (lacZYA-argF)U169  
 $\phi$ 80dlacZ $\Delta$ (M15)]. All basic DNA procedures were done  
essentially as described (39). DNA fragments were iso-  
lated from agarose gels by the freeze-thaw method (40)  
including 3 volumes of phenol during the freezing step  
to increase yield and purity. Fragments were purified  
by benzoyl-naphthoyl-DEAE (BND) cellulose (Serva Fein-  
biochemica, Heidelberg, FRG) chromatography (41).  
Plasmids used for production of infectious RNA were  
purified by sedimentation through 1 M NaCl followed by  
banding in CsCl (39). In some cases plasmids were puri-  
fied by Qiagen chromatography (Diagen GmbH, Düsseldorf,  
FRG).

6. Site-directed oligonucleotide mutagenesis. For  
oligonucleotide mutagenesis, relevant fragments of the  
SFV cDNA clone were subcloned into M13mp18 or mp 19  
(42) and transformed (43) into DH5 $\alpha$ FIQ [endA1 hsdR1  
supE44 thi1 recA1 gyrA96 relA1  $\phi$ 80dlacZ $\Delta$ (M15)  $\Delta$ (lacZYA-  
argF)U169/F'proAB lacI<sup>q</sup> lacZ $\Delta$ (M15) Tn 5] (Bethesda  
Research Laboratories). RF DNA from these constructs  
was transformed into RZ1032 (44) [Hfr KL16 dut1 ung1  
thi1 relA1 supE44 zbd279:Tn10.], and virus grown in the  
presence of uridine to incorporate uracil residues into  
the viral genome. Single stranded DNA was isolated by  
phenol extraction from PEG precipitated phage. Oligo-  
nucleotides were synthesized on an Applied Biosystems  
380B synthesizer and purified by gel filtration over  
NAP-5 columns (Pharmacia). The oligonucleotides  
5'-CGGCCAGTGAATTCTGATTGGATCCCGGGTAATTAATTGAATTACATCCC-  
TACGCAAACG, 5'-GCGCACTATTATAGCACCGGCTCCCGGGTAATTAATT-  
GACGCAAACGTTTACGGCCGCCCGG and 5'-GCGCACTATTATAGCACCATG-  
GATCCGGGTAATTAATTGACGTTTACGGCCGCCCGGTGGCG were used to  
insert the new linker sites [BamHI-SmaI-XmaI] into the  
SFV cDNA clone. The oligonucleotides 5'-CGGCGGTCTTA-  
GATTGGTGCG and 5'-CGCGGGCGCCACCGCGGCCG were used as  
sequencing primers (SP1 and SP2) up- and downstream of

the polylinker site. Phosphorylated oligonucleotides were used in mutagenesis with Sequenase (Unites States Biochemicals, Cleveland, Ohio) as described earlier (44, 45). In vitro made RF forms were transformed into DH5 $\alpha$ F'IQ and the resulting phage isolates analyzed for the presence of correct mutations by dideoxy sequencing according to the USB protocol for using Sequenase. Finally, mutant fragments were reinserted into the full-length SFV cDNA clone. Again, the presence of the appropriate mutations was verified by sequencing from the plasmid DNA. Deletion of the 6K region has been described elsewhere.

7. In vitro transcription. SpeI linearized plasmid DNA was used as template for in vitro transcription. RNA was synthesized at 37°C for 1 h in 10-50  $\mu$ l reactions containing 40 mM Tris-HCl (pH 7.6), 6 mM spermidine-HCl, 5 mM dithiothreitol (DTT), 100  $\mu$ g/ml of nuclease free BSA, 1 mM each of ATP, CTP and UTP, 500  $\mu$ M of GTP, 1 unit/ $\mu$ l of RNasin and 100-500 units/ml of SP6 RNA polymerase. For production of capped transcripts (46), the analogs m<sup>7</sup>G(5')ppp(5')G or m<sup>7</sup>G(5')ppp(5')A were included in the reaction at 1 mM. For quantitation of RNA production, trace amounts of [ $\alpha$ -<sup>32</sup>P]-UTP (Amersham) was included in the reactions and incorporation measured from trichloroacetic acid precipitates. When required, DNA or RNA was digested at 37°C for 10 min by adding DNase 1 or RNase A at 10 units/ $\mu$ g template or 20  $\mu$ g/ml respectively.

8. RNA transfection. Transfection of BHK monolayer cells by the DEAE-Dextran method was done as described previously (47). For transfection by electroporation, RNA was added either directly from the in vitro transcription reaction or diluted with transcription buffer containing 5 mM DTT and 1 unit/ $\mu$ l of RNasin. Cells were trypsinized, washed once with complete BHK-cell medium and once with ice-cold PBS (without MgCl<sub>2</sub> and CaCl<sub>2</sub>) and finally resuspended in PBS to give 10<sup>7</sup> cells/ml. Cells

were either used directly or stored (in BHK medium) on ice over night. For electroporation, 0.5 ml of cells were transferred to a 0.2 cm cuvette (BioRad), 10-50  $\mu$ l of RNA solution added and the solution mixed by inverting the cuvette. Electroporation was performed at room temperature by two consecutive pulses at 1.5 kV/25  $\mu$ F using a BioRad Gene Pulser apparatus with its pulse controller unit set at maximum resistance. After incubation for 10 min, the cells were diluted 1:20 in complete BHK-cell medium and transferred onto tissue culture plates. For plaque assays, the electroporated cells were plated together with about  $3 \times 10^5$  fresh cells per ml and incubated at 37°C for 2 h, then overlaid with 1.8 % low melting point agarose in complete BHK-cell medium. After incubation at 37°C for 48 h, plaques were visualized by staining with neutral red.

9. Gel electrophoresis. Samples for sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) were prepared and run on 12 % separating gels with a 5 % stacking gel as previously described (48). For resolving the 6K peptide, a 10 % - 20 % linear acrylamide gradient gel was used. Gels were fixed in 10 % acetic acid - 30 % methanol for 30 min before exposing to Kodak XAR-5 film. When a gel was prepared for fluorography (49), it was washed after fixation for 30 min in 30 % methanol and then soaked in 1M sodium salicylate - 30 % methanol for 30 min before drying. Nucleic acids were run on agarose gels using 50 mM Tris-borate - 2.5 mM  $\text{Na}_2\text{EDTA}$  as buffer. For staining 0.2  $\mu$ g/ml of ethidium bromide was included in the buffer and gel during the run.

#### Example 1

In this example a full-length SFV cDNA clone is prepared and placed in a plasmid containing the SP6 RNA polymerase promoter to allow in vitro transcription of full-length and infectious transcripts. This plasmid which is designated pSP6-SFV4 has been deposited on 28

NOV 1991 at PHLs Centre for Applied Microbiology & Research  
European Collection of Animal Cell Cultures, Porton  
Down, Salisbury, Wiltshire, U.K., and given the provisional accession number 91112826.

As illustrated in Fig. 4A-C the strategy for construction the SFV clone was to prime cDNA synthesis on several positions along the template RNA downstream of suitable restriction endonuclease sites defined by the known nucleotide sequence of the SFV RNA molecule. Virus RNA was isolated by phenol-chloroform extraction from purified virus (obtainable among others from the Arbovirus collection in Yale University, New Haven, USA) and used as template for cDNA synthesis as previously described (50). First strand synthesis was primed at three positions, using 5'-TTTCTCGTAGTTCTCTCTC-GTC as primer-1 (SFV coordinate 2042-2062) and 5'-GTTA-TCCCAGTGGTTGTTCTCGTAATA as primer-2 (SFV coordinate 3323-3349) and an oligo-dT<sub>12-18</sub> as primer -3 (3' end of SFV) Fig. 4A).

Second strand synthesis was preceded by hybridization of the oligonucleotide 5'-ATGGCGGATGTGTGACATACACGACGCC (identical to the 28 first bases of the genome sequence of SFV) to the first strand cDNA. After completion of second strand synthesis cDNA was trimmed and in all cases except in the case of the primer-1 reaction, the double-stranded adaptor 5'-AATTCAGCTTGC GGCCGCACTAGT / GTTCGAACGCCGCGTGATCA-3' (5'-sticky-EcoRI-HindIII-NotI-XmaIII-SpeI-blunt-3') was added and the cDNA cloned into EcoRI cleaved pTZ18R (Pharmacia, Sweden) as described (51). The cloning of the 5' end region was done in a different way. Since SFV contains a HindIII site at position 1947, cDNA primed with primer-1 should contain this area and therefore HindIII could be used to define the 3' end of that cDNA. To obtain a restriction site at the very 5' end of the SFV, cDNA was cloned into SmaI-HindIII cut pGEM1 (Promega Biotec.,

Madison, WI). Since the SFV genome starts with the sequence 5'-ATGG, ligation of this onto the blunt CCC-3' end of the SmaI site created an NcoI site C'CATGG. Although the SFV sequence contains 3 NcoI sites, none of these are within the region preceding the HindIII site, and thus these 5' end clones could be further subcloned as NcoI-HindIII fragments into a vector especially designed for this purpose (see below). The original cDNA clones in pGEM1 were screened by restriction analysis and all containing inserts bigger than 1500 bp were selected for further characterization by sequencing directly from the plasmid into both ends of the insert, using SP6 or T7 sequencing primers. The SFV 5'-end clones in pTZ18R were sequenced using lac sequencing primers. To drive in vitro synthesis of SFV RNA the SP6 promoter was used. Cloning of the SFV 5' end in front of this promoter without adding too many foreign nucleotides required that a derivative of pGEM1 had to be constructed. Hence, pGEM1 was opened at EcoRI and Bal31 deletions were created, the DNA blunted with T4 DNA polymerase and an NcoI oligonucleotide (5'-GCCATGGC) added. The clones obtained were screened by colony hybridization (39) with the oligonucleotide 5'-GGTGACACTATAGCCATGGC designed to pick up (at suitable stringency) the variants that had the NcoI sequence immediately at the transcription initiation site of the SP6 promoter (G underlined). Since the Bal31 deletion had removed all restriction sites of the multicloning site of the original plasmid, these were restored by cloning a PvuI-NcoI fragment from the new variant into another variant of pGEM1 (pDH101) that had an NcoI site inserted at its HindIII position in the polylinker. This created the plasmid pDH201. Finally, the adaptor used for cloning the SFV cDNA was inserted into pDH201 between the EcoRI and PvuII sites to create plasmid pPLH211 (Fig. 4B). This plasmid was then used as recipient for SFV cDNA fragments in the assembly of the

full-length clone by combining independent overlapping subclones using these sites. The fragments and the relevant restriction sites used to assemble the full-length clone, pSP6-SFV4, are depicted in (Fig. 4A). For the 5'-end, the selected fragment contained the proper SFV sequence 5'-ATGG, with one additional G-residue in front. When this G-residue was removed it reduced transcription efficiency from SP6 but did not affect infectivity of the in vitro made RNA. Thus, the clone used for all subsequent work contains the G-residue at the 5' end. For the 3'-end of the clone, a cDNA fragment containing 69 A-residues was selected. By inclusion of the unique SpeI site at the 3'-end of the cDNA, the plasmid can be linearized to allow for runoff transcription in vitro giving RNA-carrying 70 A-residues. Fig. 4C shows the 5' and 3' border sequences of the SFV cDNA clone. The general outline how to obtain and demonstrate infectivity of the full-length SFV RNA is depicted in Fig. 6. The complete nucleotide sequence of the pSP6-SFV4 SP6 transcript together with the amino acid sequences of the nonstructural and the structural polyproteins is shown in Fig. 5.

Typically, about 5  $\mu$ g of RNA per 100 ng of template was obtained using 10 units of polymerase, but the yield could be increased considerably by the use of more enzyme. The conditions slightly differ from those reported earlier for the production of infectious transcripts of alphaviruses (52) (47). A maximum production of RNA was obtained with rNTP concentrations at 1 mM. However, since infectivity also is dependent on the presence of a 5'cap structure optimal infectivity was obtained when the GTP concentration in the transcription reaction was halved. This drop had only a marginal effect on the amounts of RNA produced but raised the specific infectivity by a factor of 3 (data not shown).

The cDNA sequence shown in Fig. 5 has been used in the following examples. However, sequences having one



or a few nucleotides, which differ from those shown in Fig. 5, could also be useful as vectors, even if these might be less efficient as illustrated above with the SFV cDNA sequence lacking the first 5'-G nucleotide in Fig. 5.

Example 2.

In this example the construction of SFV DNA expression vectors is disclosed.

The cDNA clone coding for the complete genome of SFV obtained in Example 1 was used to construct a SFV DNA expression vector by deletion of the coding region of the 26S structural genes to make way for heterologous inserts. However, the nonstructural coding region, which is required for the production of the nsP1-4 replicase complex is preserved. RNA replication is dependent on short 5' (nt 1-247) (53, 54, 55) and 3' (nt 11423-11441) sequence elements (56, 57), and therefore, also these had to be included in the vector construct, as had the 26S promoter just upstream of the C gene (17, 18).

As is shown in Fig. 7, first, the XbaI (6640)-NsiI (8927) fragment from the SFV cDNA clone pSP6-SFV4 from Example 1 was cloned into pGEM7Zf(+) (Promega Corp., WI, USA) (Step A). From the resulting plasmid, pGEM7Zf(+)-SFV, the EcoRI fragment (SFV coordinates 7391 and 88746) was cloned into M13mp19 to insert a BamHI - XmaI - SmaI polylinker sequence immediately downstream from the 26S promoter site using site-directed mutagenesis (step B). Once the correct mutants had been verified by sequencing from M13 ssDNA (single stranded), the EcoRI fragments were reinserted into pGEM7Zf(+)-SFV (step C) and then cloned back as XbaI-NsiI fragments into pSP6-SFV4 (step D). To delete the major part of the cDNA region coding for the structural proteins of SFV, these plasmids were then cut with AsuII (7783) and NdeI (11033), blunted using Klenow fragment in the presence of all four nucleotides, and religated to create the

final vectors designated pSFV1, pSFV2 and pSFV3, respectively (step E). The vectors retain the promoter region of the 26S subgenomic RNA and the last 49 amino acids of the E1 protein as well as the complete non-coding 3' end of the SFV genome.

In the vectors the subgenomic (26S) protein coding portion has been replaced with a polylinker sequence allowing the insertional cloning of foreign cDNA sequences under the 26S promoter. As is shown in Fig. 8 these three vectors have the same basic cassette inserted downstream from the 26S promoter, i.e. a polylinker (BamHI-SmaI-XmaI) followed by a translational stop-codons in all three reading frames. The vectors differ as to the position where the polylinker cassette has been inserted. In pSFV1 the cassette is situated 31 bases downstream of the 26S transcription initiation site. The initiation motive of the capsid gene translation is identical to the consensus sequence (58). Therefore, this motive has been provided for in pSFV2, where it is placed immediately after the motive of the capsid gene. Finally, pSFV3 has the cassette placed immediately after the initiation codon (AUG) of the capsid gene. Sequencing primers (SP) needed for checking both ends of an insert have been designed to hybridize either to the 26S promoter region (SP1), or to the region following the stop codon cassette (SP2).

Note that the 26S promoter overlaps with the 3'-end of the nsP4 coding region. For pSFV2, the cloning site is positioned immediately after the translation initiation site of the SFV capsid gene. For pSFV3, the cloning site is positioned three nucleotides further downstream, i.e. immediately following to the initial AUG codon of the SFV capsid gene. The three translation stop codons following the polylinker are boxed. The downstream sequencing primer (SP1) overlaps with the 26S promoter, and the upstream sequencing primer (SP2)

overlaps the XmaIII site.

### Example 3

In this example an in vivo packaging system encompassing helper virus vector constructs is prepared. .

5       The system allows SFV variants defective in structural protein functions, or recombinant RNAs derived from the expression vector construct obtained in Example 2, to be packaged into infectious virus particles. Thus, this system allows recombinant RNAs to be introduced into cells by normal infection. The helper vector, called pSFV-Helper1, is constructed by deleting the region between the restriction endo-  
10       nuclease sites AccI (308) and AccI (6399) of pSP6-SFV4 obtained in Example 1 by cutting and religation as shown in Fig. 7, step F. The vector retains the 5' and  
15       3' signals needed for RNA replication. Since almost the complete nsP region of the Helper vector is deleted, RNA produced from this construct will not replicate in the cell due to the lack of a functional replicase  
20       complex. As is shown in Fig. 9, after transcription in vitro of pSFV1-recombinant and helper cDNAs, helper RNA is cotransfected with the pSFV1 - recombinant derivative, the helper construct providing the structural proteins needed to assemble new virus particles, and  
25       the recombinant providing the nonstructural proteins needed for RNA replication, SFV particles comprising recombinant genomes being produced. The cotransfection is preferably produced by electroporation as is disclosed in Example 6 and preferably BHK cells are used  
30       as host cells.

35       To package the RNA a region at the end of nsP1 is required, an area which has been shown to bind capsid protein (57, 59). Since the Helper lacks this region, RNA derived from this vector will not be packaged and hence, transfections with recombinant and Helper produces only virus particles that carry recombinant-derived RNA. It follows that these viruses cannot be

passaged further and thus provide a one-step virus stock. The advantage is that infections with these particles will not produce any viral proteins.

Example 4

5           This example illustrates the construction of variants of the full-length SFV cDNA clone from Example 1 that allow insertion of foreign DNA sequences encoding foreign epitopes, and the production of recombinant (chimaeric) virus carrying said foreign epitopes as  
10           integral parts of the p62, E2 or E1 spike proteins.

          To this end, a thorough knowledge of the function, topology and antigenic structure of the E2 and E1 envelope proteins has been of the essence. Earlier  
15           studies on the pathogenicity of alphaviruses have shown that antibodies against E2 are type-specific and have good neutralizing activity while those against E1 generally are group-specific and are nonneutralizing  
(5). However, not until recently have antigenic sites of the closely related alphaviruses SFV, Sindbis, and  
20           Ross River been mapped and correlated to the level of amino acid sequence (60, 61, 62, 63). These studies have shown that the most dominant sites in question are at amino acid positions 216, 234 and 246-251 of the SFV E2 spike protein. Interestingly, these three sites are  
25           exactly the same as the ones predicted by computer analysis. In the present example domain 246-251 was used, since this area has a highly conserved structure and hydropathy profile within the group of alpha-viruses. Insertion of a gene encoding a foreign epitope  
30           into the 246-251 region of the pSP6-SFV4 p62 protein yields particles with one new epitope on each heterodimer, i.e. 240 copies.

          To create a unique restriction endonuclease site that would allow specific insertion of foreign epitopes into  
35           the E2 portion of the SFV genome, a BamHI site was inserted by site directed mutagenesis using the oligonucleotide 5'-GATCGGCCTAGGAGCCGAGAGCCC.

Example 5

In this example a conditionally lethal variant of SFV is constructed from the SFV cDNA obtained in Example 1, which variant carries a mutation in the p62 protein resulting in a noncleavable form of said protein, with the result that this variant as such cannot infect new host cells, unless first cleaved with exogenously added protease.

As illustrated in Fig. 10, this construct can be advantageously used as a vaccine carrier for foreign epitopes, since this form of the virus cannot enter new host cells although assembled with wild type efficiency in transfected cells. The block can be overcome by trypsin treatment of inactive virus particles. This converts the particle into a fully entry-competent form which can be used for amplification of this virus variant stock.

Once activated the SFV variant will enter cells normally through the endocytic pathway and start infection. Viral proteins will be made and budding takes place at the plasma membrane. However, all virus particles produced will be of inactive form and the infection will thus cease after one round of replication. The reason for the block in infection proficiency is a mutation which has been introduced by site directed mutagenesis into the cleavage site of p62. This arginine to leucine substitution (at amino acid position 66 of the E3 portion of the p62 protein) changes the consensus features of the cleavage site so that it will not be recognized by the host cell proteinase that normally cleaves the p62 protein to the E2 and E3 polypeptides during transport to the cell surface. Instead, only exogenously added trypsin will be able to perform this cleavage, which in this case occurs at the arginine residue 65 immediately preceding the original cleavage site. As this cleavage regulates the activation of the entry function potential of the

virus by controlling the binding of the entry spike subunit, the virus particle carrying only uncleaved p62 will be completely unable to enter new host cells.

The creation of the cleavage deficient mutation E2 has been described earlier (29). An Asu1 - Nsu1 fragment spanning this region was then isolated and cloned into the full-length cDNA clone pSP6-SFV4.

#### Example 6

In this example transfection of BHK cells with SFV RNA molecules transcribed in vitro from full-length cDNA from Example 1 or variants thereof or the SFV vectors from Example 2, which comprise exogenous DNA, is disclosed. The transfection is carried out by electroporation which is shown to be very efficient at optimized conditions.

BHK cells were transfected with the above SFV RNA molecules by electroporation and optimal conditions were determined by varying parameters like temperature, voltage, capacitance, and number of pulses. Optimal transfection was obtained by 2 consecutive pulses of 1.5 kV at 25  $\mu$ F, under which negligible amounts of cells were killed. It was found that it was better to keep the cells at room temperature than at 0°C during the whole procedure. Transfection by electroporation was also measured as a function of input RNA. As expected, an increase in transfection frequency was not linearly dependent on RNA concentration, and about 2  $\mu$ g of cRNA were needed to obtain 100 % transfection.

On comparison with conventional transfection, this is a great improvement. For example, with DEAE-Dextran transfection optimally, only 0.2 % of the cells were transfected.

#### Example 7

This example illustrates heterologous gene expression driven by the SFV vector, pSFV1 from Example 2, for genes encoding the 21 kD cytoplasmic mouse dihydro-folate reductase (dhfr), the 90 kD membrane protein

human transferrin receptor (TR), and finally the 14 kD secretory protein chicken lysozyme. The dhfr gene was isolated from pGEM2-dhfr (64) as a BamHI-HindIII fragment blunted with Klenow fragment and inserted into SmaI-cut pSFV1. The transferrin receptor gene was first cloned from pGEM1-TR (64, 65) as an XbaI-EcoRI fragment into pGEM7ZF(+) and subsequently from there as a BamHI fragment into pSFV1. Finally, a BamHI fragment from pGEM2 carrying the lysozyme gene (21) was cloned into pSFV1.

To study the expression of the heterologous proteins, in vitro-made RNA of the dhfr and TR constructs was electroporated into BHK cells. RNA of wild type SFV was used as control. At different time points post electroporation (p.e.) cells were pulse-labeled for 10 min followed by a 10 min chase, whereafter the lysates were analyzed by gel electrophoresis and autoradiography. The results are shown in Figure 11. More specifically, BHK cells were transfected with RNAs of wild type SFV, pSFV1-dhfr, and pSFV1-TR, pulse-labeled at 3, 6, 9, 12, 15 and 24 h p.e. Equal amounts of lysate were run on a 12 % gel. The 9 h sample was also used in immunoprecipitation (IP) of the SFV, the dhfr and the transferrin receptor proteins. Cells transfected with pSFV1-lysozyme were pulse-labeled at 9 h p.e. and then chased for the times (hours) indicated. An equal portion of lysate or medium was loaded on the 13,5 % gel. IP represents immunoprecipitation from the 1 h chase lysate sample. The U-lane is lysate of labeled but untransfected cells. At 3 h p.e. hardly any exogenous proteins were made, since the incoming RNA starts with minus strand synthesis which does not peak until about 4-5 h p.e. (5). At this time point, almost all labeled proteins were of host origin. In contrast, at 6 h p.e. the exogenous proteins were synthesized with great efficiency, and severe inhibition of host protein synthesis was evident. This was even more striking at 9 h

p.e., when maximum shut down had been reached. Efficient production of the heterologous proteins continued up to 24 h p.e., after which production slowed down (data not shown), indicating that the cells had entered a stationary phase.

5 Since chicken lysozyme is a secretory protein, its expression was analyzed both from cell lysates and from the growth medium. Cells were pulse-labeled at 9 h p.e. and then chased up to 8 h. The results are shown in Fig. 11. Although lysozyme was slowly secreted, almost  
10 all labeled material was secreted to the medium during the chase.

#### Example 8

15 This example illustrates the present in vivo packaging system.

In vitro-made RNA of pSFV1-TR was mixed with Helper RNA at different ratios and these mixtures were co-transfected into BHK cells. Cells were grown for 24 h after which the culture medium was collected and the virus particles pelleted by ultracentrifugation. The  
20 number of infectious units (i.u.) was determined by immunofluorescence. It was found that a 1:1 ratio of Helper and recombinant most efficiently produced infectious particles, and on the average  $5 \times 10^6$  cells yielded  $2.5 \times 10^9$  i.u. The infectivity of the virus  
25 stock was tested by infecting BHK cells at different multiplicities of infection (m.o.i.). In Fig. 11 the results for expression of human transferrin receptor in BHK cells after infection by such in vivo packaged  
30 particles carrying pSFV1-TR recombinant RNA is shown to the lower right. 200  $\mu$ l of virus diluted in MEM (including 0.5% BAS and 2 mM glutamine) was overlaid on cells to give m.o.i. values ranging from 5 to 0.005. After 1 h at 37°C, complete BHK medium was added and  
35 growth continued for 9 h, at which time a 10 min pulse (100  $\mu$ Ci  $^{35}$ S-methionine/ml) and 10 min chase was performed, and the cells dissolved in lysis buffer. 10



5  $\mu$ l out of the 300  $\mu$ l lysate (corresponding to 30,000 cells) was run on the 10 % gel, and the dried gel was exposed for 2 h at  $-70^{\circ}\text{C}$ . Due to the high expression level, only 3,000 cells are needed to obtain a distinct band on the autoradiograph with an over night exposure.

Thus, it was found that efficient protein production and concomitant hos protein shut-off occurred at about 1 i.u. per cell. Since one SFV infected cell produces on the average  $10^8$  capsid protein molecules, it follows that a virus stock produced from a single electropora-  
10 tion can be used to produce  $10^{17}$  protein molecules equaling about 50 mg of protein.

From the foregoing experimental results it is obvious that the present invention is related to very useful  
15 and efficient expression system which lacks several of the disadvantages of the hitherto existing expression system. The major advantages of the present system are shortly summarized as follows:

- 20 (1) High titre recombinant virus stocks can be produced in one day by one transfection experiment. There is no need for selection/screening, plaque purification and amplification steps. This is appreciated since an easy production of recombinant virus is  
25 especially important in experiments where the phenotypes of large series of mutants have to be characterized.
- 30 (2) The recombinant virus stock is free from helper virus since only the recombinant genome but not the helper genome contains a packaging signal.
- 35 (3) The recombinant virus can be used to infect the recombinant genome in a "natural" and nonleakey way into a large variety of cells including insect and most higher euoaryotic cell types. Such a wide host range is very useful for an expressions system

especially when cell-type-specific posttranslational modification reactions are required for the activity of the expressed protein.

- (4) The level of protein expression obtained is extremely high, the level corresponding to those of the viral proteins during infection. There is also a host cell protein shut-off which makes it possible to follow the foreign proteins clearly in cell lysates without the need for antibody mediated antigen concentration. This will facilitate DNA expression experiments in cell biology considerably. Furthermore, problems of interference by the endogenous counter part to an expressed protein (i.e. homo-oligomerization reactions) can be avoided.

#### Example 9

This example illustrates epitope carriers.

A very important example where vaccine development is of the utmost importance concerns the acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus HIV-1 (66, 67). So far, all attempts to produce an efficient vaccine against HIV-1 have failed, although there was a very recent report that vaccination with disrupted SIV-1 (Simian immunodeficiency virus) to a certain extent may give protection against infections of that virus (68). However, development of safe and effective vaccine against HIV-1 will be very difficult due to the biological properties of the virus. In the present example one epitope of HIV-1 was inserted into an antigenic domain of the E2 protein of SFV. The epitope used is located in glycoprotein gp120 of HIV-1, spanning amino acids 309-325. This forms the variable loop of HIV-1 and is situated immediately after an N-glycosylated site.

A chimera was constructed where the 309-325 epitope of HIV was inserted into the BamHI site using cassette

insertion of ready-made oligonucleotides encoding the HIV epitope. The required base substitutions at the BamHI site did not lead to any amino acid changes in the vector, although two amino acids (Asp and Glu) changed places. This change did not have any deleterious effect since in vitro made vector RNA induced cell infection with wild type efficiency. Fig. 12 shows the sequences in the area of interest in the epitope carrier. In preliminary experiments, it has been shown that chimaeric proteins were produced. The proteins can be immunoprecipitated with anti-HIV antibodies. It is to be expected that these are also used for production of chimaeric virus particles that can be used for vaccine preparation against HIV. Such particles are shown in Fig. 12, lower part.

#### List of references

- 1) Bishop, D.H.L. (1990). Gene expression using insect cells and viruses. In current Opinion in Biotechnology, Vol. 1, Rosenberg, M., and Moss, B., eds. (London: Current Opinion Ltd.), pp. 62-67.
- 2) Moss, B. (1990). Regulation of Vaccinia virus transcription. Ann. Rev. Biochem. 59, 661-688.
- 3) Moss, B, and Flexner, C. (1989). Vaccinia virus expression vectors. Ann. N.Y. Acad Sci. 569, 86-103.
- 4) Garoff, H., Kondor-Koch, C., and Riedel, H. (1982). Structure and assembly of alphaviruses. Curr. Top. Microbiol. Immunol. 99, 1-50.
- 5) Strauss, E.G., and Strauss, J.H. (1986).

Structure and replication of the alphavirus genome. In *The Togaviridae and Flaviviridae*, Vol. Schlesinger, S.S., and Schlesinger, M.J., eds (New York: Plenum Press), pp. 35-90.

- 6) Garoff, H., Frischauf, A.-M., Simons, K., Lehrach, H., and Delius, H. (1980). Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature* 288, 236-241.
- 7) Takkinen, K. (1986). Complete nucleotide sequence of the nonstructural protein genes of Semliki forest virus. *Nucl. Acids Res.* 14, 5667-5682.
- 8) de Groot, R.J., Hardy, W.R., Shirako, Y., and Strauss, J.H. (1990). Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing in vivo. *EMBO J.* 9, 2631-2638.
- 9) Hahn, Y.S., Strauss, E.G., and Strauss, J.H. (1989b). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and C to nonstructural proteins. *J. Virol.* 63, 3142-3150.
- 10) Mi, S., Durbin, R., Huang, H.V., Rice, C.M., and Stollar, V. (1989). Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. *Virology* 170, 385-391.
- 11) Ding, M., and Schlesinger, M.J. (1989). Evidence that Sindbis virus nsP2 is an auto-

protease which processes the virus non-structural polyprotein. *Virology* 171, 280-284.

- 12) Hardy, W.R., and Strauss, J.H. (1989). Processing the nonstructural polyproteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in cis and in trans. *J. Virol.* 63, 4653-4664.
- 13) Li, G., La Starza, M.W., Hardy, W.R., Strauss, J.H., and Rice, C.M. (1990). Phosphorylation of Sindbis virus nsP3 in vivo and in vitro.
- 14) Peränen, J., Takkinen, K., Kalkkinen, N., and Kääriäinen, L. (1988). Semliki Forest virus-specific nonstructural protein nsP3 is a phosphoprotein. *J. Gen. Virol.* 69, 2165-2178.
- 15) Hahn, Y.S., Grakoui, A., Rice, C.M., Strauss, E.G., and Strauss, J.H. (1989a). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4.
- 16) Sawicki, D.L., Barkhimer, D.B. Sawicki, S.G., Rice, C.M., and Schlesinger, S. (1990). Temperature sensitive shut-off of alphavirus minus strand RNA synthesis maps to a nonstructural protein, nsP4. *Virology* 174, 43-52.
- 17) Grakoui, A., Levis, R., Raju, R., Huang, H.V., and Rice, C.M. (1989). A cis-acting mutation in the Sindbis virus junction region which affects subgenomic RNA synthesis. *J. Virol.* 63, 5216-5227.
- 18) Levis, R., Schlesinger, S., and Huang, H.V.

- (1990). Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. *J. Virol.* 64, 1726-1733.
- 19) Schlesinger, S.S., and Schlesinger, M.J. (1986). Formation and assembly of alphavirus glycoproteins. In *The Togaviridae and Flaviviridae*, Vol. Schlesinger, S.S., and Schlesinger, M.J., eds. (New York: Plenum Press), pp.121-148.
- 20) Hahn, C.S., and Strauss, J.H. (1990). Site-directed mutagenesis of the proposed catalytic amino acids of the Sindbis virus capsid protein autoprotease. *J. Virol.* 64, 3069-3073.
- 21) Melançon, P., and Garoff, H. (1987). Processing of the Semliki Forest virus structural polyprotein; Role of the capsid protease. *J. Virol.* 61, 1301-1309.
- 22) Bonatti, S., Migliaccio, G., Blobel, G., and Walter, P (1984). Role of the signal recognition particle in the membrane assembly of Sindbis viral glycoprotein. *Eur. J. Biochem.* 140, 499-502.
- 23) Garoff, H., Simons, K., and Dobberstein, B. (1978). Assembly of Semliki Forest virus membrane glycoproteins in the membrane of the endoplasmic reticulum in vitro. *J. Mol. Biol.* 124, 587-600.
- 24) Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U., and Liljeström, P. (1990). The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. *J. Cell*

Biol. 111, 867-876.

- 25) Melançon, P., and Garoff, H. (1986). Reinitiation of translocation in the Semliki Forest virus structural polyprotein: Identification of the signal for the E1 glycoprotein. *EMBO J.* 5, 1551-1560.
- 26) Lobigs, M., Zhao, H., and Garoff, H. (1990b). Function of Semliki Forest virus E3 peptide in virus assembly: Replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. *J. Virol.* 64, 4346-4355.
- 27) de Curtis, I., and Simons, K. (1988). Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. *Proc. Natl. Acad. Sci. USA*, 85, 8052-8056.
- 28) Helenius, A., Kielian, M., Mellman, I., and Schmid, S. (1989). Entry of enveloped viruses into their host cells. In *Cell Biology of Virus Entry, Replication, and Pathogenesis*, Vol. 90, Compans, R. W., Helenius, A., and Oldstone, M.B.A., eds. (New York: Alan R. Liss, Inc.), pp. 145-161.
- 29) Lobigs, M., and Garoff, H. (1990). Fusion function of the Semliki Forest virus spike is activated by proteolytic cleavage of the envelope glycoprotein p62. *J. Virol.* 64, 1233-1240.
- 30) Lobigs, M., Wahlberg, J.M., and Garoff, H. (1990a). Spike protein oligomerization control

- of Semliki Forest virus fusion. J. Virol. 64, 5214-5218.
- 31) Wahlberg, J.M., Boere, W.A., and Garoff, H. (1989). The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to mildly acidic pH during virus maturation. J. Virol. 63, 4991-4997.
- 32) Ziemiecki, A., Garoff, H., and Simons, K. (1980). Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. J. Gen. Virol. 50, 111-123.
- 33) Fuller, S.D. (1987). The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. Cell 48, 923-934.
- 34) Wengler, G. (1980). Effects of alphaviruses on host cell macromolecular synthesis. In The Togaviruses, Vol. Schlesinger, R. W., eds. (New York: Academic Press, Inc.), pp. 459-472.
- 35) Stollar, V. (1980). Defective interfering alphaviruses. In The Togaviruses, Vol. Schlesinger, R.W., eds. (New York: Academic Press), pp. 427-457.
- 36) Boere, W.A.M., Harmsen, T., Vinje, J., Benaissa-Trouw, B.J., Kraaijeveld, C.A., and Snippe, H. (1984). Identification of distinct antigenic determinants on Semliki Forest virus by using monoclonal antibodies with different antiviral activities. J. Virol. 52, 575-582.
- 37) Greiser-Wilke, I., Moennig, V., Kaaden, O.-R.,



- and Figueiredo, L.T.M. (1989). Most alpha-viruses share a conserved epitopic region on their nucleocapsid protein. *J. Gen. Virol.* 70, 743-748.
- 38) Kondor, K.C., Bravo, R., Fuller, S.D., Cutler, D., and Garoff, H. (1985). Exocytotic pathways exist to both the apical and the basolateral cell surface of the polarized epithelial cell MDCK. *Cell* 43, 297-306.
- 39) Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual.* (Cold Spring Harbor: Cold spring Harbor Laboratory Press).
- 40) Benson, S.A. (1984). A rapid procedure for isolation of DNA fragments from agarose gels. *Bio Techniques* 2, 66-68.
- 41) Silhavy, T.J., Berman, M.L., and Enquist, L.W. (1984). *Experiments with Gene Fusions.* (New York: Cold Spring Harbor Laboratory Press).
- 42) Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33, 103-119.
- 43) Chung, C.T., and Miller, R.T. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucl. Acids Res.* 16, 3580.
- 44) Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific muta-

- genesis without phenotypic selection. *Meth. Enzymol.* 154, 367-382.
- 45) Su, T.-Z., and El-Gewely, M.R. (1988). A multisite-directed mutagenesis using T7 DNA polymerase: application for reconstructing a mammalian gene. *Gen* 69, 81-89.
- 46) Krieg, P.A., and Melton, D.A. (1987). In vitro RNA synthesis with SP6 RNA polymerase. *Meth. Enzymol.* 155, 397-415.
- 47) Rice, C.M., Levis, R., Strauss, J.H., and Huang, H.V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: Mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *J. Virol.* 61, 3809-3819.
- 48) Cutler, D.F., and Garoff, H. (1986). Mutants of the membrane-binding region of Semliki Forest virus E2 protein. I. Cell surface transport and fusogenic activity. *J. Cell Biol.* 102, 889-901.
- 49) Chamberlain, J.P. (1979). Fluorographic detection of radioactivity in polyacrylamide gels with watersoluble fluor, sodium salicylate. *Anal. Biochem.* 98, 132-135.
- 50) Gubler, U., and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* 25, 263-269.
- 51) Haymerle, H., Herz, J., Bressan, G.M., Frank, R., and Stanley, K.K. (1986). Efficient construction of cDNA libraries in plasmid expres-

- sion vectors using an adaptor strategy. Nucl. Acids Res. 14, 8615-8124.
- 52) Davis, N.L., Willis, L.V., Smith, J.F., and Johnston, R.E. (1989). In vitro synthesis of infectious Venezuelan Equine Encephalitis virus RNA from a cDNA clone: Analysis of a viable deletion mutant. Virology 171, 189-204.
- 53) Niesters, H.G., and Strauss, J.H. (1990a). Defined mutations in the 5' nontranslated sequence of Sindbis virus RNA. J. Virol. 64, 4162-4168.
- 54) Niesters, H.G.M., and Strauss, J.H. (1990b). Mutagenesis of the conserved 51-nucleotide region of Sindbis virus. J. Virol. 64, 1639-1647.
- 55) Tsiang, M., Weiss, B.G., and Schlesinger, S. (1988). Effects of 5'-terminal modifications on the biological activity of defective interfering RNAs of Sindbis virus. J. Virol. 62, 47-53.
- 56) Kuhn, R.J., Hong, Z., and Strauss, J.H. (1990). Mutagenesis of the 3' nontranslated region of Sindbis virus RNA. J. Virol. 64, 1465-1476.
- 57) Levis, R., Weiss, B.G., Tsiang, M., Huang, H., and Schlesinger, S. (1986). Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell 44, 137-145.
- 58) Kozak, M. (1989). The scanning model for translation: an update. J. Cell Biol. 108, 229-241.

- 59) Weiss, B., Nitschko, H., Ghattas, I., Wright, R., and Schlesinger, S. (1989). Evidence for specificity in the encapsidation of Sindbis virus RNAs. *J. Virol.* 63, 5310-5318.
- 60) Davis NL, Pence DF, Meyer WJ, Schmaljohn AL and Johnston RE (1987). Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. *Virology* 161:101-108.
- 61) Mendoza QP, Stanley J and Griffin DE (1988). Monoclonal antibodies to the E1 and E2 glycoproteins of Sindbis virus: Definition of epitopes and efficiency of protection from fatal encephalitis. *J. Gen. Virol.* 70:3015-3022.
- 62) Vрати S, Fernon CA, Dalgarno L and Weir RC (1988). Location of a major antigenic site involved in Ross River virus neutralization. *Virology* 162:346-353.
- 63) Grosfeld H, Velan B, Leitner M. Cohen S, Lustig S, Lachmi B and Shafferman A (1989). Semliki Forest virus E2 envelope epitopes induce a nonneutralizing humoral response which protects mice against lethal challenge. *J. Virol.* 63:3416-3422.
- 64) Zerial, M., Melançon, P., Schneider, C., and Garoff, H. (1986). The transmembrane segment of the human transferrin receptor functions as a signal peptide. *EMBO J.* 5, 1543-1550.
- 65) Schneider, C., Owen, M.J., Banville, D., and Williams, J.G. (1984). Primary structure of

human transferrin receptor deduced from the mRNA sequence. Nature 311, 675-678.

- 66) Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalki JA, Whitehorn EA, Baumeister K, Ivanoff L, Petteway SR, Pearson ML, Lautenberger JA, Papas TS, Ghrayeb J, Chang NT, Gallo RC and Wong-Staal F (1985). Complete nucleotide sequence of the AIDS virus, HTLVIII. Nature 313:277-284.
- 67) AIDS (1988). Sci.Am. 259. A single-topic issue on HIV biology.
- 68) Desrosiers RC, Wyand MS, Kodama T, Ringler DJ, Arthur LO, Sehgal PK, Letvin NL, King NW and Daniel MD (1989). Vaccine protection against simian immunodeficiency virus infection.
- 69) Ginsberg H, Brown F, Lerner RA and Chanoch RM (1988). Vaccines 1988. New chemical and genetic approaches to vaccination, Cold Spring Harbor Laboratory, 396 pp.
- 70) Burke KL, Dunn G, Ferguson M, Minor PD and Almond JW (1988). Antigen chimeras of poliovirus as potential new vaccines. Nature 332:81-82.
- 71) Colbere-Garapin F, Christodoulou C, Crainic R, Garapin A-C and Candrea A (1988). Addition of a foreign oligopeptide to the major capsid protein of poliovirus. Proc. Natl. Acad. Sci. USA 85:8668-8672.
- 72) Evans DJ, McKeating J, Meredith JM, Burke KL, Katrak K, John A, Ferguson M, Minor PD, Weiss

RA and Almond JW (1989). An engineered polio-virus chimaera elicits broadly reactive HIV-1 neutralizing antibodies. Nature 339:385-388.

-----

Claims

1. An RNA molecule derived from an alphavirus RNA genome and capable of efficient infection of animal host cells, which RNA molecule comprises the complete alphavirus RNA genome regions, which are essential to replication of the said alphavirus RNA, and further comprises an exogenous RNA sequence capable of expressing its function in said host cell, said exogenous RNA sequence being inserted into a region of the RNA molecule which is non-essential to replication thereof.
2. The RNA of claim 1, wherein the said alphavirus is Semliki Forest virus (SFV).
3. The RNA of claim 1 or 2, wherein the exogenous RNA sequence encodes a protein, a polypeptide or a peptide sequence defining an exogenous antigenic epitope or determinant.
4. The RNA of claim 3 wherein the exogenous RNA sequence encodes an epitope sequence of a structural protein of an immunodeficiency virus inclusive of the human immunodeficiency virus (HIV) types.
5. The RNA of any preceding claim, wherein the alphavirus derived RNA molecule regions comprise a 5' terminal portion, the coding region(s) for non structural proteins required for RNA replication, the subgenome promoter region and a 3' terminal portion of said viral RNA.
6. The RNA of claim 2, 3 or 5, wherein the exogenous RNA sequence encodes a foreign polypeptide or protein and is integrated into the SFV subgenomic 26S RNA substituting deleted parts thereof.
7. The RNA of claim 2, 3, 4 or 5, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide sequence and is located in a region of the RNA coding for structural alphavirus proteins enabling the exogenous RNA to be expressed as said viral epitope as part of the matured virus particle.
8. The RNA of claim 2, 3, 4 or 5, wherein the exogenous RNA sequence encodes a foreign viral epitopic peptide sequence inserted into the p62 spike precursor subunit encoding region of the SFV genome.
9. An RNA expression vector comprising the RNA of any preceding claim packaged into infectious particles comprising the RNA within the alphavirus nucleocapsid and surrounded by membrane with alphavirus spike proteins.

10. The vector of claim 9, wherein the RNA has a total size corresponding to the wild type alphavirus RNA genome or deviating therefrom to an extent compatible with package of the RNA into the infectious particles.

5 11. DNA transcription vector comprising a cDNA having one strand complementary to the RNA of any of claims 1 to 8.

12. A DNA expression vector comprising a full-length or partial cDNA complementary to alphavirus RNA or parts thereof and located immediately downstream of the SP6 RNA polymerase promoter and having a 5'ATGG or 5'GATGG or any other 5' terminus and a TTTCCA<sub>69</sub>ACTAGT or any other 3' terminus.

13. The vector of claim 12 having portions of the viral cDNA deleted, the deletions comprising the complete or part of the region(s) encoding the virus structural proteins, and further comprising an integrated polylinker region, which may correspond to BamHI-SmaI-XmaI, inserted at a location which enables an exogenous DNA fragment encoding a foreign polypeptide or protein to be inserted into the vector cDNA for subsequent expression in an animal host cell.

20 14. The vector of claim 12 or 13 wherein the alphavirus is SFV.

15. The vector of claim 12 or 14 comprising full-length cDNA and further comprising an exogenous DNA fragment encoding a foreign epitopic peptide sequence or antigenic determinant inserted into a region of the viral structural proteins.

16. The vector of claim 15 wherein the exogenous DNA fragment is inserted into the p62 spike precursor subunit encoding region of the SFV cDNA.

17. The vector of any preceding claim comprising an SFV derived cDNA which carries a conditionally lethal SFV mutation in the region encoding the p62 cleavage site, a cellularly uncleavable but extracellularly cleavable form of p62 being expressed.

18. The vector of claim 13 comprising SFV-derived cDNA, the vector being pSFV1, pSFV2 or pSFV3 having a structure as shown in Fig. 8.

19. An RNA transcript derived from transcription of the DNA-vector of any of claims 12-18 carrying an exogenous DNA fragment.

20. A method to produce the vector of claim 9 or 10



wherein the alphavirus derived RNA lacks part of or the complete region(s) encoding the structural viral proteins, the method comprising cotransfection of animal host cells with the RNA transcript of claim 19, wherein the alphavirus RNA lacks  
5 part(s) of or the complete region(s) encoding the viral structural proteins, with helper RNA transcribed in vitro from a helper DNA vector and culturing the host cells.

21. The method of claim 20 wherein the cotransfection is produced by electroporation of the host cells.

10 22. Helper vector for use in the method according to claim 20 or 21, said vector being comprised of the DNA vector of claim 12 wherein the regions encoding non structural virus proteins are almost completely deleted, including sequences encoding RNA signals for packaging of RNA into nucleocapsid  
15 particles, but the 5' and 3' signals needed for RNA replication and the region encoding the promoter for the structural sub-genome are in addition to those encoding the structural region preserved.

23. Helper vector of claim 22 wherein the cDNA has its  
20 origin from SFV and the deletion extends from the AccI (308) to the AccI (6399) restriction endonuclease site of the full-length cDNA vector of claim 12.

24. Helper vector of claim 22 and 23 where the structural region contains the mutation described in claim 17 or another  
25 conditionally lethal mutation.

25. The method of claim 20 wherein cells transformed to produce helper RNA according to claims 20, 22 or 23 are transfected with RNA transcript of claim 19.

26. A host cell of animal origin transformed with the RNA  
30 of any of claims 1-8, the DNA transcription vector of claims 11 or the DNA vector of any of claims 12-18 carrying an exogenous DNA fragment.

27. The host cell of claim 26 wherein the cell is an avian, a mammalian, a reptilian, an amphibian, an insecticidal  
35 or a fish cell.

28. The host cell of claim 27 which is the hamster BHK cell.

29. A method to produce the transformed host cell of claim 26, 27 or 28 comprising transfection of the cell with the  
40 RNA of any of claim 1-8, with the cDNA of claim 11 or of any of

claims 12-18 carrying an exogenous DNA fragment or infection of the cell with the infectious viral particles of claim 9 or 10.

30. The method of claim 29 wherein the transfection is produced by electroporation of the host cell.

5 31. A method for the production of a polypeptide or protein comprising infection of animal host cells with infectious particles according to claim 9 or 10, containing exogenous RNA encoding said polypeptide or protein and produced according to method of claim 20 or 21, culturing the said  
10 transformed cells to express the exogenous RNA and isolation and purification of the product formed by said expression.

15 32. A method for the production of a polypeptide or protein comprising in vitro transcription of the cDNA of the vector of any of claims 11-18 carrying an exogenous DNA fragment coding for the polypeptide or protein, transfection of animal host cells with the produced RNA transcript, transformed animal host cells being obtained harbouring the RNA transcript, culturing the said transformed cells to express the exogenous RNA and isolation and purification of the product formed by  
20 said expression.

33. The method of claim 32 wherein the vector cDNA is comprised of the cDNA of the vector of claim 17 carrying the exogenous DNA fragment.

25 34. An antigen consisting of a chimaeric alphavirus having an exogenous epitopic peptide sequence or antigenic determinant inserted into its structural proteins.

35. The antigen of claim 34 wherein the chimaeric alphavirus is derived from SFV.

30 36. The antigen of claim 34 or 35, wherein the exogenous epitopic peptide sequence is comprised of an epitopic peptide sequence derived from a structural protein of a virus belonging to the immunodeficiency virus class inclusive of the human immunodeficiency virus types.

35 37. Vaccine preparation comprising the antigen of claim 34, 35 or 36 as immunizing component.

38. Vaccine of claim 37 wherein the chimaeric alphavirus is attenuated by comprising the conditionally lethal SFV mutation of claim 17, an amber (stop codon) a temperature sensitive mutation or other mutation in its genome.

40 39. A method for the production of an antigen of claim

34, 35 or 36 comprising

a) in vitro transcription of the cDNA of the vector of any of claims 11-18 carrying an exogenous DNA fragment encoding the foreign epitopic peptide sequence or antigenic determinant and transfection of animal host cells with the produced RNA transcript, or

b) transfection of animal host cells with the said cDNA of the above step a),

culturing the transfected cells and recovering the chimaeric alphavirus antigen.

40. The method of claim 32, 33 or 39 wherein the transfection is produced by electroporation of the host cell.

41. A method for the production of an antigen in an organism by using in vivo infection with infectious particles according to claim 9 or 10 containing exogenous RNA encoding an exogenous epitopic peptide sequence or antigenic determinant, and produced according the claim 20 or 21.

-----



2/33

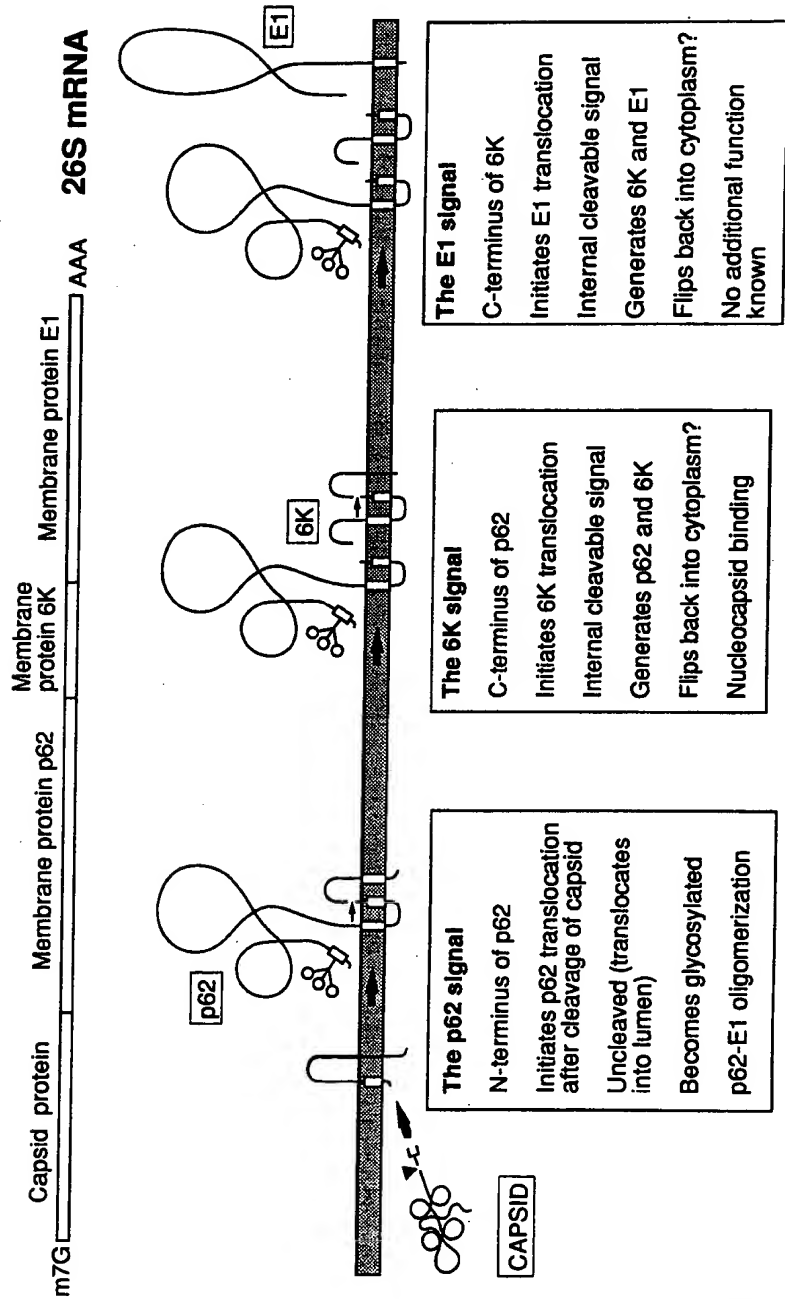


Figure 2

SUBSTITUTE SHEET

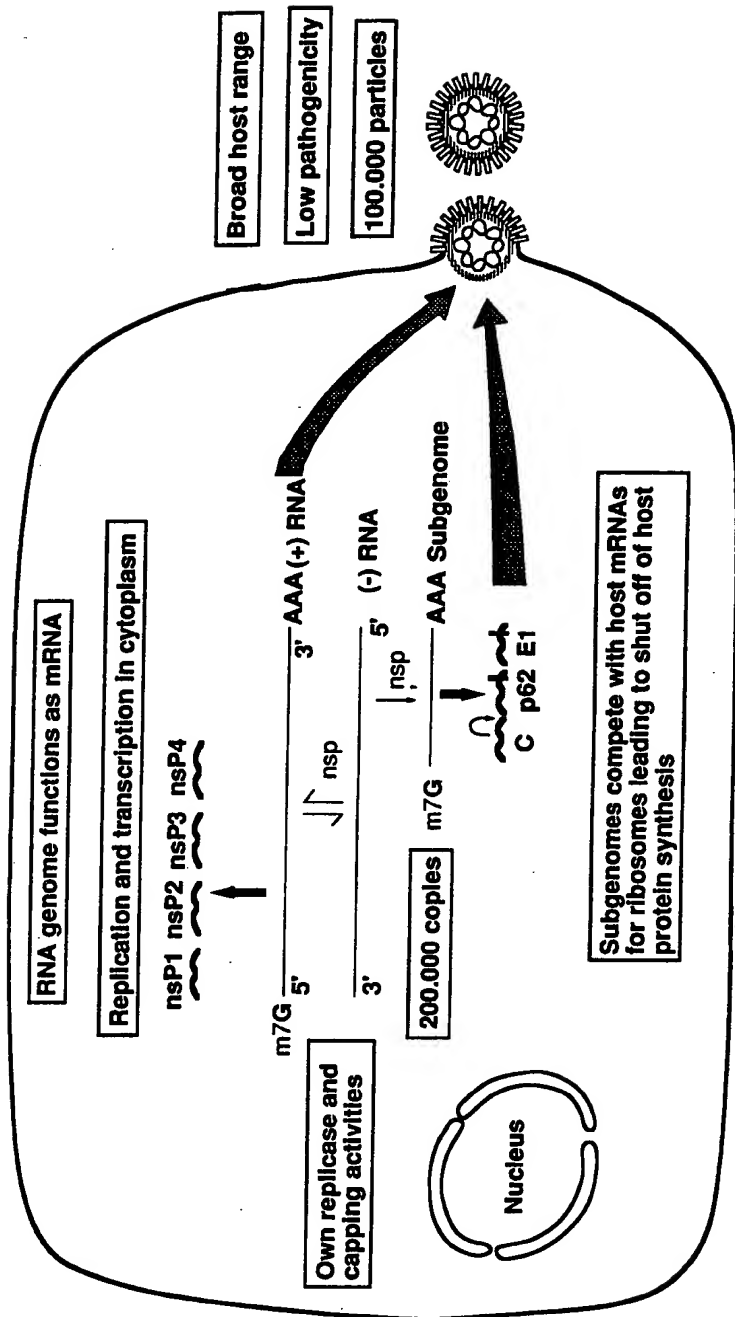
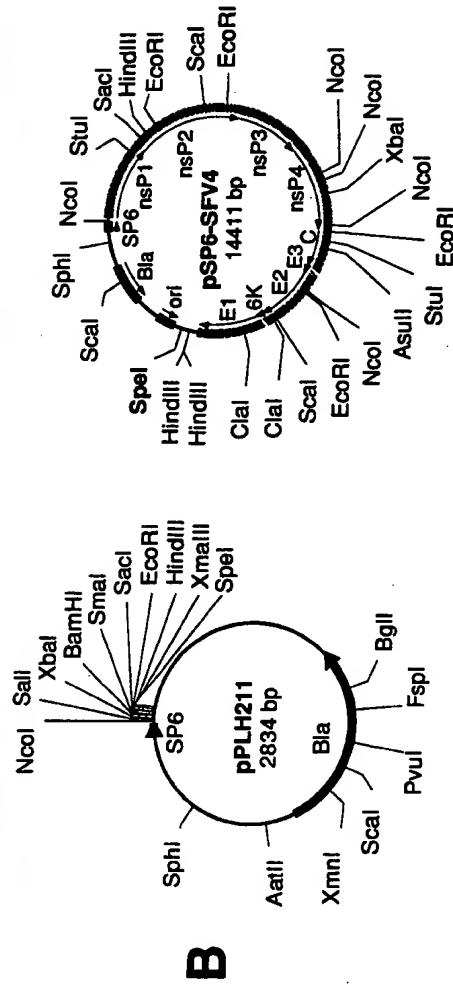
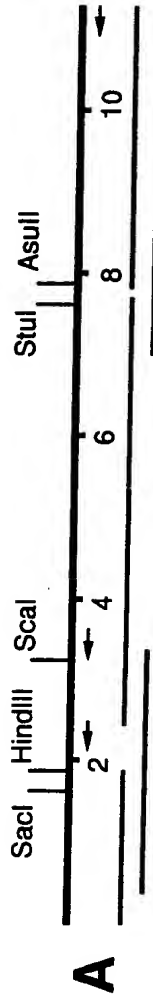


Figure 3

**4/33**



## Figure 4

**SUBSTITUTE SHEET**

Figure 5 (1)

5/33

GATGCGGAT GTGTGACATA CACGACGCCA AAAGATTTTG TTCCAGCTCC TGCCACCTCC 60  
 GCTACGCGAG AGATTAACCA CCCACG ATG GCC GCC AAA GTG CAT GTT GAT ATT 113  
 Met Ala Ala Lys Val His Val Asp Ile  
 5

GAG GCT GAC AGC CCA TTC ATC AAG TCT TTG CAG AAG GCA TTT CCG 158  
 Glu Ala Asp Ser Pro Phe Ile Lys Ser Leu Gln Lys Ala Phe Pro  
 10 15 20

TCG TTC GAG GTG GAG TCA TTG CAG GTC ACA CCA AAT GAC CAT GCA 203  
 Ser Phe Glu Val Glu Ser Leu Gln Val Thr Pro Asn Asp His Ala  
 25 30 35

AAT GCC AGA GCA TTT TCG CAC CTG GCT ACC AAA TTG ATC GAG CAG 248  
 Asn Ala Arg Ala Phe Ser His Leu Ala Thr Lys Leu Ile Glu Gln  
 40 45 50

GAG ACT GAC AAA GAC ACA CTC ATC TTG GAT ATC GGC AGT GCG CCT 293  
 Glu Thr Asp Lys Asp Thr Leu Ile Leu Asp Ile Gly Ser Ala Pro  
 55 60 65

TCC AGG AGA ATG ATG TCT ACG CAC AAA TAC CAC TGC GTA TGC CCT 338  
 Ser Arg Arg Met Met Ser Thr His Lys Tyr His Cys Val Cys Pro  
 70 75 80

ATG CGC AGC GCA GAA GAC CCC GAA AGG CTC GAT AGC TAC GCA AAG 383  
 Met Arg Ser Ala Glu Asp Pro Glu Arg Leu Asp Ser Tyr Ala Lys  
 85 90 95

AAA CTG GCA GCG GCC TCC GGG AAG GTG CTG GAT AGA GAG ATC GCA 428  
 Lys Leu Ala Ala Ala Ser Gly Lys Val Leu Asp Arg Glu Ile Ala  
 100 105 110

GGA AAA ATC ACC GAC CTG CAG ACC GTC ATG GCT ACG CCA GAC GCT 473  
 Gly Lys Ile Thr Asp Leu Gln Thr Val Met Ala Thr Pro Asp Ala  
 115 120 125

GAA TCT CCT ACC TTT TGC CTG CAT ACA GAC GTC ACG TGT CGT ACG 518  
 Glu Ser Pro Thr Phe Cys Leu His Thr Asp Val Thr Cys Arg Thr  
 130 135 140

GCA GCC GAA GTG GCC GTA TAC CAG GAC GTG TAT GCT GTA CAT GCA 563  
 Ala Ala Glu Val Ala Val Tyr Gln Asp Val Tyr Ala Val His Ala  
 145 150 155

CCA ACA TCG CTG TAC CAT CAG GCG ATG AAA GGT GTC AGA ACG GCG 608  
 Pro Thr Ser Leu Tyr His Gln Ala Met Lys Gly Val Arg Thr Ala  
 160 165 170

TAT TGG ATT GGG TTT GAC ACC ACC CCG TTT ATG TTT GAC GCG CTA 653  
 Tyr Trp Ile Gly Phe Asp Thr Thr Pro Phe Met Phe Asp Ala Leu  
 175 180 185

SUBSTITUTE SHEET



Figure 5 (2)

6/33

GCA GGC GCG TAT CCA ACC TAC GCC ACA AAC TGG GCC GAC GAG CAG	698
Ala Gly Ala Tyr Pro Thr Tyr Ala Thr Asn Trp Ala Asp Glu Gln	
190	195 200
GTG TTA CAG GCC AGG AAC ATA GGA CTG TGT GCA GCA TCC TTG ACT	743
Val Leu Gln Ala Arg Asn Ile Gly Leu Cys Ala Ala Ser Leu Thr	
205	210 215
GAG GGA AGA CTC GGC AAA CTG TCC ATT CTC CGC AAG AAG CAA TTG	788
Glu Gly Arg Leu Gly Lys Leu Ser Ile Leu Arg Lys Lys Gln Leu	
220	225 230
AAA CCT TGC GAC ACA GTC ATG TTC TCG GTA GGA TCT ACA TTG TAC	833
Lys Pro Cys Asp Thr Val Met Phe Ser Val Gly Ser Thr Leu Tyr	
235	240 245
ACT GAG AGC AGA AAG CTA CTG AGG AGC TGG CAC TTA CCC TCC GTA	878
Thr Glu Ser Arg Lys Leu Leu Arg Ser Trp His Leu Pro Ser Val	
250	255 260
TTC CAC CTG AAA GGT AAA CAA TCC TTT ACC TGT AGG TGC GAT ACC	923
Phe His Leu Lys Gly Lys Gln Ser Phe Thr Cys Arg Cys Asp Thr	
265	270 275
ATC GTA TCA TGT GAA GGG TAC GTA GTT AAG AAA ATC ACT ATG TGC	968
Ile Val Ser Cys Glu Gly Tyr Val Val Lys Lys Ile Thr Met Cys	
280	285 290
CCC GGC CTG TAC GGT AAA ACG GTA GGG TAC GCC GTG ACG TAT CAC	1013
Pro Gly Leu Tyr Gly Lys Thr Val Gly Tyr Ala Val Thr Tyr His	
295	300 305
CGC GAG GGA TTC CTA GTG TGC AAG ACC ACA GAC ACT GTC AAA GGA	1058
Ala Glu Gly Phe Leu Val Cys Lys Thr Thr Asp Thr Val Lys Gly	
310	315 320
GAA AGA GTC TCA TTC CCT GTA TGC ACC TAC GTC CCC TCA ACC ATC	1103
Glu Arg Val Ser Phe Pro Val Cys Thr Tyr Val Pro Ser Thr Ile	
325	330 335
TGT GAT CAA ATG ACT GGC ATA CTA GCG ACC GAC GTC ACA CCG GAG	1148
Cys Asp Gln Met Thr Gly Ile Leu Ala Thr Asp Val Thr Pro Glu	
340	345 350
GAC GCA CAG AAG TTG TTA GTG GGA TTG AAT CAG AGG ATA GTT GTG	1193
Asp Ala Gln Lys Leu Leu Val Gly Leu Asn Gln Arg Ile Val Val	
355	360 365
AAC GGA AGA ACA CAG CGA AAC ACT AAC ACG ATG AAG AAC TAT CTG	1238
Asn Gly Arg Thr Gln Arg Asn Thr Asn Thr Met Lys Asn Tyr Leu	
370	375 380
CTT CCG ATT GTG GCC GTC GCA TTT AGC AAG TGG GCG AGG GAA TAC	1283
Leu Pro Ile Val Ala Val Ala Phe Ser Lys Trp Ala Arg Glu Tyr	
385	390 395

SUBSTITUTE SHEET

Figure 5 (3)

7/33

AAG GCA GAC CTT GAT GAT GAA AAA CCT CTG GGT GTC CGA GAG AGG 1328  
 Lys Ala Asp Leu Asp Asp Glu Lys Pro Leu Gly Val Arg Glu Arg  
 400 405 410

TCA CTT ACT TGC TGC TGC TTG TGG GCA TTT AAA ACG AGG AAG ATG 1373  
 Ser Leu Thr Cys Cys Cys Leu Trp Ala Phe Lys Thr Arg Lys Met  
 415 420 425

CAC ACC ATG TAC AAG AAA CCA GAC ACC CAG ACA ATA GTG AAG GTG 1418  
 His Thr Met Tyr Lys Lys Pro Asp Thr Gln Thr Ile Val Lys Val  
 430 435 440

CCT TCA GAG TTT AAC TCG TTC GTC ATC CCG AGC CTA TGG TCT ACA 1463  
 Pro Ser Glu Phe Asn Ser Phe Val Ile Pro Ser Leu Trp Ser Thr  
 445 450 455

GGC CTC GCA ATC CCA GTC AGA TCA CGC ATT AAG ATG CTT TTG GCC 1508  
 Gly Leu Ala Ile Pro Val Arg Ser Arg Ile Lys Met Leu Leu Ala  
 460 465 470

AAG AAG ACC AAG CGA GAG TTA ATA CCT GTT CTC GAC GCG TCG TCA 1553  
 Lys Lys Thr Lys Arg Glu Leu Ile Pro Val Leu Asp Ala Ser Ser  
 475 480 485

GCC AGG GAT GCT GAA CAA GAG GAG AAG GAG AGG TTG GAG GCC GAG 1598  
 Ala Arg Asp Ala Glu Gln Glu Glu Lys Glu Arg Leu Glu Ala Glu  
 490 495 500

CTG ACT AGA GAA GCC TTA CCA CCC CTC GTC CCC ATC GCG CCG GCG 1643  
 Leu Thr Arg Glu Ala Leu Pro Pro Leu Val Pro Ile Ala Pro Ala  
 505 510 515

GAG ACG GGA GTC GTC GAC GTC GAC GTT GAA GAA CTA GAG TAT CAC 1688  
 Glu Thr Gly Val Val Asp Val Asp Val Glu Glu Leu Glu Tyr His  
 520 525 530

GCA GGT GCA GGG GTC GTG GAA ACA CCT CGC AGC GCG TTG AAA GTC 1733  
 Ala Gly Ala Gly Val Glu Thr Pro Arg Ser Ala Leu Lys Val  
 535 540 545

ACC GCA CAG CCG AAC GAC GTA CTA CTA GGA AAT TAC GTA GTT CTG 1778  
 Thr Ala Gln Pro Asn Asp Val Leu Leu Gly Asn Tyr Val Val Leu  
 550 555 560

TCC CCG CAG ACC GTG CTC AAG AGC TCC AAG TTG GCC CCC GTG CAC 1823  
 Ser Pro Gln Thr Val Leu Lys Ser Ser Lys Leu Ala Pro Val His  
 565 570 575

CCT CTA GCA GAG CAG GTG AAA ATA ATA ACA CAT AAC GGG AGG GCC 1868  
 Pro Leu Ala Glu Gln Val Lys Ile Ile Thr His Asn Gly Arg Ala  
 580 585 590

GGC GGT TAC CAG GTC GAC GGA TAT GAC GGC AGG GTC CTA CTA CCA 1913  
 Gly Gly Tyr Gln Val Asp Gly Tyr Asp Gly Arg Val Leu Leu Pro  
 595 600 605

SUBSTITUTE SHEET

Figure 5 (4)

8/33

TGT GGA TCG GCC ATT CCG GTC CCT GAG TTT CAA GCT TTG AGC GAG	1958
Cys Gly Ser Ala Ile Pro Val Pro Glu Phe Gln Ala Leu Ser Glu	
610 615 620	
AGC GCC ACT ATG GTG TAC AAC GAA AGG GAG TTC GTC AAC AGG AAA	2003
Ser Ala Thr Met Val Tyr Asn Glu Arg Glu Phe Val Asn Arg Lys	
625 630 635	
CTA TAC CAT ATT GCC GTT CAC GGA CCG TCG CTG AAC ACC GAC GAG	2048
Leu Tyr His Ile Ala Val His Gly Pro Ser Leu Asn Thr Asp Glu	
640 645 650	
GAG AAC TAC GAG AAA GTC AGA GCT GAA AGA ACT GAC GCC GAG TAC	2093
Glu Asn Tyr Glu Lys Val Arg Ala Glu Arg Thr Asp Ala Glu Tyr	
655 660 665	
GTG TTC GAC GTA GAT AAA AAA TGC TGC GTC AAG AGA GAG GAA GCG	2138
Val Phe Asp Val Asp Lys Lys Cys Cys Val Lys Arg Glu Glu Ala	
670 675 680	
TCG GGT TTG GTG TTG GTG GGA GAG CTA ACC AAC CCC CCG TTC CAT	2183
Ser Gly Leu Val Leu Val Gly Glu Leu Thr Asn Pro Pro Phe His	
685 690 695	
GAA TTC GCC TAC GAA GGG CTG AAG ATC AGG CCG TCG GCA CCA TAT	2228
Glu Phe Ala Tyr Glu Gly Leu Lys Ile Arg Pro Ser Ala Pro Tyr	
700 705 710	
AAG ACT ACA GTA GTA GGA GTC TTT GGG GTT CCG GGA TCA GGC AAG	2273
Lys Thr Thr Val Val Gly Val Phe Gly Val Pro Gly Ser Gly Lys	
715 720 725	
TCT GCT ATT ATT AAG AGC CTC GTG ACC AAA CAC GAT CTG GTC ACC	2318
Ser Ala Ile Ile Lys Ser Leu Val Thr Lys His Asp Leu Val Thr	
730 735 740	
AGC GGC AAG AAG GAG AAC TGC CAG GAA ATA GTT AAC GAC GTG AAG	2363
Ser Gly Lys Lys Glu Asn Cys Gln Glu Ile Val Asn Asp Val Lys	
745 750 755	
AAG CAC CGC GGG AAG GGG ACA AGT AGG GAA AAC AGT GAC TCC ATC	2408
Lys His Arg Gly Lys Gly Thr Ser Arg Glu Asn Ser Asp Ser Ile	
760 765 770	
CTG CTA AAC GGG TGT CGT CGT GCC GTG GAC ATC CTA TAT GTG GAC	2453
Leu Leu Asn Gly Cys Arg Arg Ala Val Asp Ile Leu Tyr Val Asp	
775 780 785	
GAG GCT TTC GCT TGC CAT TCC GGT ACT CTG CTG GCC CTA ATT GCT	2498
Glu Ala Phe Ala Cys His Ser Gly Thr Leu Leu Ala Leu Ile Ala	
790 795 800	
CTT GTT AAA CCT CGG AGC AAA GTG GTG TTA TGC GGA GAC CCC AAG	2543
Leu Val Lys Pro Arg Ser Lys Val Val Leu Cys Gly Asp Pro Lys	
805 810 815	

SUBSTITUTE SHEET

Figure 5 (5)

9/33

CAA TGC GGA TTC TTC AAT ATG ATG CAG CTT AAG GTG AAC TTC AAC	2588
Gln Cys Gly Phe Phe Asn Met Met Gln Leu Lys Val Asn Phe Asn	
820 825 830	
CAC AAC ATC TGC ACT GAA GTA TGT CAT AAA AGT ATA TCC AGA CGT	2633
His Asn Ile Cys Thr Glu Val Cys His Lys Ser Ile Ser Arg Arg	
835 840 845	
TGC ACG CGT CCA GTC ACG GCC ATC GTG TCT ACG TTG CAC TAC GGA	2678
Cys Thr Arg Pro Val Thr Ala Ile Val Ser Thr Leu His Tyr Gly	
850 855 860	
GGC AAG ATG CGC ACG ACC AAC CCG TGC AAC AAA CCC ATA ATC ATA	2723
Gly Lys Met Arg Thr Thr Asn Pro Cys Asn Lys Pro Ile Ile Ile	
865 870 875	
GAC ACC ACA GGA CAG ACC AAG CCC AAG CCA GGA GAC ATC GTG TTA	2768
Asp Thr Thr Gly Gln Thr Lys Pro Lys Pro Gly Asp Ile Val Leu	
880 885 890	
ACA TGC TTC CGA GGC TGG GCA AAG CAG CTG CAG TTG GAC TAC CGT	2813
Thr Cys Phe Arg Gly Trp Ala Lys Gln Leu Gln Leu Asp Tyr Arg	
895 900 905	
GGA CAC GAA GTC ATG ACA GCA GCA GCA TCT CAG GGC CTC ACC CGC	2858
Gly His Glu Val Met Thr Ala Ala Ala Ser Gln Gly Leu Thr Arg	
910 915 920	
AAA GGG GTA TAC GCC GTA AGG CAG AAG GTG AAT GAA AAT CCC TTG	2903
Lys Gly Val Tyr Ala Val Arg Gln Lys Val Asn Glu Asn Pro Leu	
925 930 935	
TAT GCC CCT GCG TCG GAG CAC GTG AAT GTA CTG CTG ACG CGC ACT	2948
Tyr Ala Pro Ala Ser Glu His Val Asn Val Leu Leu Thr Arg Thr	
940 945 950	
GAG GAT AGG CTG GTG TGG AAA ACG CTG GCC GGC GAT CCC TGG ATT	2993
Glu Asp Arg Leu Val Trp Lys Thr Leu Ala Gly Asp Pro Trp Ile	
955 960 965	
AAG GTC CTA TCA AAC ATT CCA CAG GGT AAC TTT ACG GCC ACA TTG	3038
Lys Val Leu Ser Asn Ile Pro Gln Gly Asn Phe Thr Ala Thr Leu	
970 975 980	
GAA GAA TGG CAA GAA GAA CAC GAC AAA ATA ATG AAG GTG ATT GAA	3083
Glu Glu Trp Gln Glu Glu His Asp Lys Ile Met Lys Val Ile Glu	
985 990 995	
GGA CCG GCT GCG CCT GTG GAC GCG TTC CAG AAC AAA GCG AAC GTG	3128
Gly Pro Ala Ala Pro Val Asp Ala Phe Gln Asn Lys Ala Asn Val	
1000 1005 1010	
TGT TGG GCG AAA AGC CTG GTG CCT GTC CTG GAC ACT GCC GGA ATC	3173
Cys Trp Ala Lys Ser Leu Val Pro Val Leu Asp Thr Ala Gly Ile	
1015 1020 1025	

SUBSTITUTE SHEET

Figure 5 (6)

10/33

AGA TTG ACA GCA GAG GAG TGG AGC ACC ATA ATT ACA GCA TTT AAG 3218  
 Arg Leu Thr Ala Glu Glu Trp Ser Thr Ile Ile Thr Ala Phe Lys  
 1030 1035 1040

GAG GAC AGA GCT TAC TCT CCA GTG GTG GCC TTG AAT GAA ATT TGC 3263  
 Glu Asp Arg Ala Tyr Ser Pro Val Val Ala Leu Asn Glu Ile Cys  
 1045 1050 1055

ACC AAG TAC TAT GGA GTT GAC CTG GAC AGT GGC CTG TTT TCT GCC 3308  
 Thr Lys Tyr Tyr Gly Val Asp Leu Asp Ser Gly Leu Phe Ser Ala  
 1060 1065 1070

CCG AAG GTG TCC CTG TAT TAC GAG AAC AAC CAC TGG GAT AAC AGA 3353  
 Pro Lys Val Ser Leu Tyr Tyr Glu Asn Asn His Trp Asp Asn Arg  
 1075 1080 1085

CCT GGT GGA AGG ATG TAT GGA TTC AAT GCC GCA ACA GCT GCC AGG 3398  
 Pro Gly Gly Arg Met Tyr Gly Phe Asn Ala Ala Thr Ala Ala Arg  
 1090 1095 1100

CTG GAA GCT AGA CAT ACC TTC CTG AAG GGG CAG TGG CAT ACG GGC 3443  
 Leu Glu Ala Arg His Thr Phe Leu Lys Gly Gln Trp His Thr Gly  
 1105 1110 1115

AAG CAG GCA GTT ATC GCA GAA AGA AAA ATC CAA CCG CTT TCT GTG 3488  
 Lys Gln Ala Val Ile Ala Glu Arg Lys Ile Gln Pro Leu Ser Val  
 1120 1125 1130

CTG GAC AAT GTA ATT CCT ATC AAC CGC AGG CTG CCG CAC GCC CTG 3533  
 Leu Asp Asn Val Ile Pro Ile Asn Arg Arg Leu Pro His Ala Leu  
 1135 1140 1145

GTG GCT GAG TAC AAG ACG GTT AAA GGC AGT AGG GTT GAG TGG CTG 3578  
 Val Ala Glu Tyr Lys Thr Val Lys Gly Ser Arg Val Glu Trp Leu  
 1150 1155 1160

GTC AAT AAA GTA AGA GGG TAC CAC GTC CTG CTG GTG AGT GAG TAC 3623  
 Val Asn Lys Val Arg Gly Tyr His Val Leu Leu Val Ser Glu Tyr  
 1165 1170 1175

AAC CTG GCT TTG CCT CGA CGC AGG GTC ACT TGG TTG TCA CCG CTG 3668  
 Asn Leu Ala Leu Pro Arg Arg Arg Val Thr Trp Leu Ser Pro Leu  
 1180 1185 1190

AAT GTC ACA GGC GCC GAT AGG TGC TAC GAC CTA AGT TTA GGA CTG 3713  
 Asn Val Thr Gly Ala Asp Arg Cys Tyr Asp Leu Ser Leu Gly Leu  
 1195 1200 1205

CCG GCT GAC GCC GGC AGG TTC GAC TTG GTC TTT GTG AAC ATT CAC 3758  
 Pro Ala Asp Ala Gly Arg Phe Asp Leu Val Phe Val Asn Ile His  
 1210 1215 1220

ACG GAA TTC AGA ATC CAC CAC TAC CAG CAG TGT GTC GAC CAC GCC 3803  
 Thr Glu Phe Arg Ile His His Tyr Gln Gln Cys Val Asp His Ala  
 1225 1230 1235

Figure 5 (7)

11/33

ATG AAG CTG CAG ATG CTT GGG GGA GAT GCG CTA CGA CTG CTA AAA 3848  
 Met Lys Leu Gln Met Leu Gly Gly Asp Ala Leu Arg Leu Leu Lys  
 1240 1245 1250

CCC GGC GGC ATC TTG ATG AGA GCT TAC GGA TAC GCC GAT AAA ATC 3893  
 Pro Gly Gly Ile Leu Met Arg Ala Tyr Gly Tyr Ala Asp Lys Ile  
 1255 1260 1265

AGC GAA GCC GTT GTT TCC TCC TTA AGC AGA AAG TTC TCG TCT GCA 3938  
 Arg Glu Ala Val Val Ser Ser Leu Ser Arg Lys Phe Ser Ser Ala  
 1270 1275 1280

AGA GTG TTG CGC CCG GAT TGT GTC ACC AGC AAT ACA GAA GTG TTC 3983  
 Arg Val Leu Arg Pro Asp Cys Val Thr Ser Asn Thr Glu Val Phe  
 1285 1290 1295

TTG CTG TTC TCC AAC TTT GAC AAC GGA AAG AGA CCC TCT ACG CTA 4028  
 Leu Leu Phe Ser Asn Phe Asp Asn Gly Lys Arg Pro Ser Thr Leu  
 1300 1305 1310

CAC CAG ATG AAT ACC AAG CTG AGT GCC GTG TAT GCC GGA GAA GCC 4073  
 His Gln Met Asn Thr Lys Leu Ser Ala Val Tyr Ala Gly Glu Ala  
 1315 1320 1325

ATG CAC ACG GCC GGG TGT GCA CCA TCC TAC AGA GTT AAG AGA GCA 4118  
 Met His Thr Ala Gly Cys Ala Pro Ser Tyr Arg Val Lys Arg Ala  
 1330 1335 1340

GAC ATA GCC ACG TGC ACA GAA GCG GCT GTG GTT AAC GCA GCT AAC 4163  
 Asp Ile Ala Thr Cys Thr Glu Ala Ala Val Val Asn Ala Ala Asn  
 1345 1350 1355

GCC CGT GGA ACT GTA GGG GAT GGC GTA TGC AGG GCC GTG GCG AAG 4208  
 Ala Arg Gly Thr Val Gly Asp Gly Val Cys Arg Ala Val Ala Lys  
 1360 1365 1370

AAA TGG CCG TCA GCC TTT AAG GGA GCA GCA ACA CCA GTG GGC ACA 4253  
 Lys Trp Pro Ser Ala Phe Lys Gly Ala Ala Thr Pro Val Gly Thr  
 1375 1380 1385

ATT AAA ACA GTC ATG TGC GGC TCG TAC CCC GTC ATC CAC GCT GTA 4298  
 Ile Lys Thr Val Met Cys Gly Ser Tyr Pro Val Ile His Ala Val  
 1390 1395 1400

GCG CCT AAT TTC TCT GCC ACG ACT GAA GCG GAA GGG GAC CGC GAA 4343  
 Ala Pro Asn Phe Ser Ala Thr Thr Glu Ala Glu Gly Asp Arg Glu  
 1405 1410 1415

TTG GCC GCT GTC TAC CGG GCA GTG GCC GCC GAA GTA AAC AGA CTG 4388  
 Leu Ala Ala Val Tyr Arg Ala Val Ala Ala Glu Val Asn Arg Leu  
 1420 1425 1430

TCA CTG AGC AGC GTA GCC ATC CCG CTG CTG TCC ACA GGA GTG TTC 4433  
 Ser Leu Ser Ser Val Ala Ile Pro Leu Leu Ser Thr Gly Val Phe  
 1435 1440 1445

SUBSTITUTE SHEET

Figure 5 (8)

12/33

AGC GGC GGA AGA GAT AGG CTG CAG CAA TCC CTC AAC CAT CTA TTC 4478  
 Ser Gly Gly Arg Asp Arg Leu Gln Gln Ser Leu Asn His Leu Phe  
 1450 1455 1460

ACA GCA ATG GAC GCC ACG GAC GCT GAC GTG ACC ATC TAC TGC AGA 4523  
 Thr Ala Met Asp Ala Thr Asp Ala Asp Val Thr Ile Tyr Cys Arg  
 1465 1470 1475

GAC AAA AGT TGG GAG AAG AAA ATC CAG GAA GCC ATT GAC ATG AGG 4568  
 Asp Lys Ser Trp Glu Lys Lys Ile Gln Glu Ala Ile Asp Met Arg  
 1480 1485 1490

ACG GCT GTG GAG TTG CTC AAT GAT GAC GTG GAG CTG ACC ACA GAC 4613  
 Thr Ala Val Glu Leu Leu Asn Asp Asp Val Glu Leu Thr Thr Asp  
 1495 1500 1505

TTG GTG AGA GTG CAC CCG GAC AGC AGC CTG GTG GGT CGT AAG GGC 4658  
 Leu Val Arg Val His Pro Asp Ser Ser Leu Val Gly Arg Lys Gly  
 1510 1515 1520

TAC AGT ACC ACT GAC GGG TCG CTG TAC TCG TAC TTT GAA GGT ACG 4703  
 Tyr Ser Thr Thr Asp Gly Ser Leu Tyr Ser Tyr Phe Glu Gly Thr  
 1525 1530 1535

AAA TTC AAC CAG GCT GCT ATT GAT ATG GCA GAG ATA CTG ACG TTG 4748  
 Lys Phe Asn Gln Ala Ala Ile Asp Met Ala Glu Ile Leu Thr Leu  
 1540 1545 1550

TGG CCC AGA CTG CAA GAG GCA AAC GAA CAG ATA TGC CTA TAC GCG 4793  
 Trp Pro Arg Leu Gln Glu Ala Asn Glu Gln Ile Cys Leu Tyr Ala  
 1555 1560 1565

CTG GGC GAA ACA ATG GAC AAC ATC AGA TCC AAA TGT CCG GTG AAC 4838  
 Leu Gly Glu Thr Met Asp Asn Ile Arg Ser Lys Cys Pro Val Asn  
 1570 1757 1580

GAT TCC GAT TCA TCA ACA CCT CCC AGG ACA GTG CCC TGC CTG TGC 4883  
 Asp Ser Asp Ser Ser Thr Pro Pro Arg Thr Val Pro Cys Leu Cys  
 1585 1590 1595

CGC TAC GCA ATG ACA GCA GAA CGG ATC GCC CGC CTT AGG TCA CAC 4928  
 Arg Tyr Ala Met Thr Ala Glu Arg Ile Ala Arg Leu Arg Ser His  
 1600 1605 1610

CAA GTT AAA AGC ATG GTG GTT TGC TCA TCT TTT CCC CTC CCG AAA 4973  
 Gln Val Lys Ser Met Val Val Cys Ser Ser Phe Pro Leu Pro Lys  
 1615 1620 1625

TAC CAT GTA GAT GGG GTG CAG AAG GTA AAG TGC GAG AAG GTT CTC 5018  
 Tyr His Val Asp Gly Val Gln Lys Val Lys Cys Glu Lys Val Leu  
 1630 1635 1640

CTG TTC GAC CCG ACG GTA CCT TCA GTG GTT AGT CCG CGG AAG TAT 5063  
 Leu Phe Asp Pro Thr Val Pro Ser Val Val Ser Pro Arg Lys Tyr  
 1645 1650 1655

SUBSTITUTE SHEET

Figure 5 (9)

13/33

GCC GCA TCT ACG ACG GAC CAC TCA GAT CGG TCG TTA CGA GGG TTT 5108  
 Ala Ala Ser Thr Thr Asp His Ser Asp Arg Ser Leu Arg Gly Phe  
 1660 1665 1670

GAC TTG GAC TGG ACC ACC GAC TCG TCT TCC ACT GCC AGC GAT ACC 5153  
 Asp Leu Asp Trp Thr Thr Asp Ser Ser Ser Thr Ala Ser Asp Thr  
 1675 1680 1685

ATG TCG CTA CCC AGT TTG CAG TCG TGT GAC ATC GAC TCG ATC TAC 5198  
 Met Ser Leu Pro Ser Leu Gln Ser Cys Asp Ile Asp Ser Ile Tyr  
 1690 1695 1700

GAG CCA ATG GCT CCC ATA GTA GTG ACG GCT GAC GTA CAC CCT GAA 5243  
 Glu Pro Met Ala Pro Ile Val Val Thr Ala Asp Val His Pro Glu  
 1705 1710 1715

CCC GCA GGC ATC GCG GAC CTG GCG GCA GAT GTG CAC CCT GAA CCC 5288  
 Pro Ala Gly Ile Ala Asp Leu Ala Ala Asp Val His Pro Glu Pro  
 1720 1725 1730

GCA GAC CAT GTG GAC CTC GAG AAC CCG ATT CCT CCA CCG CGC CCG 5333  
 Ala Asp His Val Asp Leu Glu Asn Pro Ile Pro Pro Pro Arg Pro  
 1735 1740 1745

AAG AGA GCT GCA TAC CTT GCC TCC CGC GCG GCG GAG CGA CCG GTG 5378  
 Lys Arg Ala Ala Tyr Leu Ala Ser Arg Ala Ala Glu Arg Pro Val  
 1750 1755 1760

CCG GCG CCG AGA AAG CCG ACG CCT GCC CCA AGG ACT GCG TTT AGG 5423  
 Pro Ala Pro Arg Lys Pro Thr Pro Ala Pro Arg Thr Ala Phe Arg  
 1765 1770 1775

AAC AAG CTG CCT TTG ACG TTC GGC GAC TTT GAC GAG CAC GAG GTC 5468  
 Asn Lys Leu Pro Leu Thr Phe Gly Asp Phe Asp Glu His Glu Val  
 1780 1785 1790

GAT GCG TTG GCC TCC GGG ATT ACT TTC GGA GAC TTC GAC GAC GTC 5513  
 Asp Ala Leu Ala Ser Gly Ile Thr Phe Gly Asp Phe Asp Asp Val  
 1795 1800 1805

CTG CGA CTA GGC CGC GCG GGT GCA TAT ATT TTC TCC TCG GAC ACT 5558  
 Leu Arg Leu Gly Arg Ala Gly Ala Tyr Ile Phe Ser Ser Asp Thr  
 1810 1815 1820

GGC AGC GGA CAT TTA CAA CAA AAA TCC GTT AGG CAG CAC AAT CTC 5603  
 Gly Ser Gly His Leu Gln Gln Lys Ser Val Arg Gln His Asn Leu  
 1825 1830 1835

CAG TGC GCA CAA CTG GAT GCG GTC CAG GAG GAG AAA ATG TAC CCG 5648  
 Gln Cys Ala Gln Leu Asp Ala Val Gln Glu Glu Lys Met Tyr Pro  
 1840 1845 1850

CCA AAA TTG GAT ACT GAG AGG GAG AAG CTG TTG CTG CTG AAA ATG 5693  
 Pro Lys Leu Asp Thr Glu Arg Glu Lys Leu Leu Leu Lys Met  
 1855 1860 1865

SUBSTITUTE SHEET



Figure 5 (10)

14/33

CAG ATG CAC CCA TCG GAG GCT AAT AAG AGT CGA TAC CAG TCT CGC 5738  
 Gln Met His Pro Ser Glu Ala Asn Lys Ser Arg Tyr Gln Ser Arg  
 1870 1875 1880

AAA GTG GAG AAC ATG AAA GCC ACG GTG GTG GAC AGG CTC ACA TCG 5783  
 Lys Val Glu Asn Met Lys Ala Thr Val Val Asp Arg Leu Thr Ser  
 1885 1890 1895

GGG GCC AGA TTG TAC ACG GGA GCG GAC GTA GGC CGC ATA CCA ACA 5828  
 Gly Ala Arg Leu Tyr Thr Gly Ala Asp Val Gly Arg Ile Pro Thr  
 1900 1905 1910

TAC GCG GTT CGG TAC CCC CGC CCC GTG TAC TCC CCT ACC GTG ATC 5873  
 Tyr Ala Val Arg Tyr Pro Arg Pro Val Tyr Ser Pro Thr Val Ile  
 1915 1920 1925

GAA AGA TTC TCA AGC CCC GAT GTA GCA ATC GCA GCG TGC AAC GAA 5918  
 Glu Arg Phe Ser Ser Pro Asp Val Ala Ile Ala Ala Cys Asn Glu  
 1930 1935 1940

TAC CTA TCC AGA AAT TAC CCA ACA GTG CCG TCG TAC CAG ATA ACA 5963  
 Tyr Leu Ser Arg Asn Tyr Pro Thr Val Ala Ser Tyr Gln Ile Thr  
 1945 1950 1955

GAT GAA TAC GAC GCA TAC TTG GAC ATG GTT GAC GGG TCG GAT AGT 6008  
 Asp Glu Tyr Asp Ala Tyr Leu Asp Met Val Asp Gly Ser Asp Ser  
 1960 1965 1970

TGC TTG GAC AGA GCG ACA TTC TGC CCG GCG AAG CTC CGG TGC TAC 6053  
 Cys Leu Asp Arg Ala Thr Phe Cys Pro Ala Lys Leu Arg Cys Tyr  
 1975 1980 1985

CCG AAA CAT CAT GCG TAC CAC CAG CCG ACT GTA CGC AGT GCC GTC 6098  
 Pro Lys His His Ala Tyr His Gln Pro Thr Val Arg Ser Ala Val  
 1990 1995 2000

CCG TCA CCC TTT CAG AAC ACA CTA CAG AAC GTG CTA GCG GCC GCC 6143  
 Pro Ser Pro Phe Gln Asn Thr Leu Gln Asn Val Leu Ala Ala Ala  
 2005 2010 2015

ACC AAG AGA AAC TGC AAC GTC ACG CAA ATG CGA GAA CTA CCC ACC 6188  
 Thr Lys Arg Asn Cys Asn Val Thr Gln Met Arg Glu Leu Pro Thr  
 2020 2025 2030

ATG GAC TCG GCA GTG TTC AAC GTG GAG TGC TTC AAG CGC TAT GCC 6233  
 Met Asp Ser Ala Val Phe Asn Val Glu Cys Phe Lys Arg Tyr Ala  
 2035 2040 2045

TGC TCC GGA GAA TAT TGG GAA GAA TAT GCT AAA CAA CCT ATC CGG 6278  
 Cys Ser Gly Glu Tyr Trp Glu Glu Tyr Ala Lys Gln Pro Ile Arg  
 2050 2055 2060

ATA ACC ACT GAG AAC ATC ACT ACC TAT GTG ACC AAA TTG AAA GGC 6323  
 Ile Thr Thr Glu Asn Ile Thr Thr Tyr Val Thr Lys Leu Lys Gly  
 2065 2070 2075

SUBSTITUTE SHEET

Figure 5 (11)

15/33

CCG AAA GCT GCT GCC TTG TTC GCT AAG ACC CAC AAC TTG GTT CCG	6368
Pro Lys Ala Ala Ala Leu Phe Ala Lys Thr His Asn Leu Val Pro	
2080	2085 2090
CTG CAG GAG GTT CCC ATG GAC AGA TTC ACG GTC GAC ATG AAA CGA	6413
Leu Gln Glu Val Pro Met Asp Arg Phe Thr Val Asp Met Lys Arg	
2095	2100 2105
GAT GTC AAA GTC ACT CCA GGG ACG AAA CAC ACA GAG GAA AGA CCC	6458
Asp Val Lys Val Thr Pro Gly Thr Lys His Thr Glu Glu Arg Pro	
2110	2115 2120
AAA GTC CAG GTA ATT CAA GCA GCG GAG CCA TTG GCG ACC GCT TAC	6503
Lys Val Gln Val Ile Gln Ala Ala Glu Pro Leu Ala Thr Ala Tyr	
2125	2130 2135
CTG TGC GGC ATC CAC AGG GAA TTA GTA AGG AGA CTA AAT GCT GTG	6548
Leu Cys Gly Ile His Arg Glu Leu Val Arg Arg Leu Asn Ala Val	
2140	2145 2150
TTA CGC CCT AAC GTG CAC ACA TTG TTT GAT ATG TCG GCC GAA GAC	6593
Leu Arg Pro Asn Val His Thr Leu Phe Asp Met Ser Ala Glu Asp	
2155	2160 2165
TTT GAC GCG ATC ATC GCC TCT CAC TTC CAC CCA GGA GAC CCG GTT	6638
Phe Asp Ala Ile Ile Ala Ser His Phe His Pro Gly Asp Pro Val	
2170	2175 2180
CTA GAG ACG GAC ATT GCA TCA TTC GAC AAA AGC CAG GAC GAC TCC	6683
Leu Glu Thr Asp Ile Ala Ser Phe Asp Lys Ser Gln Asp Asp Ser	
2185	2190 2195
TTG GCT CTT ACA GGT TTA ATG ATC CTC GAA GAT CTA GGG GTG GAT	6728
Leu Ala Leu Thr Gly Leu Met Ile Leu Glu Asp Leu Gly Val Asp	
2200	2205 2210
CAG TAC CTG CTG GAC TTG ATC GAG GCA GCC TTT GGG GAA ATA TCC	6773
Gln Tyr Leu Leu Asp Leu Ile Glu Ala Ala Phe Gly Glu Ile Ser	
2215	2220 2225
AGC TGT CAC CTA CCA ACT GGC ACG CGC TTC AAG TTC GGA GCT ATG	6818
Ser Cys His Leu Pro Thr Gly Thr Arg Phe Lys Phe Gly Ala Met	
2230	2235 2240
ATG AAA TCG GGC ATG TTT CTG ACT TTG TTT ATT AAC ACT GTT TTG	6863
Met Lys Ser Gly Met Phe Leu Thr Leu Phe Ile Asn Thr Val Leu	
2245	2250 2255
AAC ATC ACC ATA GCA AGC AGG GTA CTG GAG CAG AGA CTC ACT GAC	6908
Asn Ile Thr Ile Ala Ser Arg Val Leu Glu Gln Arg Leu Thr Asp	
2260	2265 2270
TCC GCC TGT GCG GCC TTC ATC GGC GAC GAC AAC ATC GTT CAC GGA	6953
Ser Ala Cys Ala Ala Phe Ile Gly Asp Asp Asn Ile Val His Gly	
2275	2280 2285

SUBSTITUTE SHEET

Figure 5 (12)

16/33

GTG ATC TCC GAC AAG CTG ATG GCG GAG AGG TGC GCG TCG TGG GTC 6998  
 Val Ile Ser Asp Lys Leu Met Ala Glu Arg Cys Ala Ser Trp Val  
 2290 2295 2300

AAC ATG GAG GTG AAG ATC ATT GAC GCT GTC ATG GGC GAA AAA CCC 7043  
 Asn Met Glu Val Lys Ile Ile Asp Ala Val Met Gly Glu Lys Pro  
 2305 2310 2315

CCA TAT TTT TGT GGG GGA TTC ATA GTT TTT GAC AGC GTC ACA CAG 7088  
 Pro Tyr Phe Cys Gly Gly Phe Ile Val Phe Asp Ser Val Thr Gln  
 2320 2325 2330

ACC GCC TGC CGT GTT TCA GAC CCA CTT AAG CGC CTG TTC AAG TTG 7133  
 Thr Ala Cys Arg Val Ser Asp Pro Leu Lys Arg Leu Phe Lys Leu  
 2335 2340 2345

GGT AAG CCG CTA ACA GCT GAA GAC AAG CAG GAC GAA GAC AGG CGA 7178  
 Gly Lys Pro Leu Thr Ala Glu Asp Lys Gln Asp Glu Asp Arg Arg  
 2350 2355 2360

CGA GCA CTG AGT GAC GAG GTT AGC AAG TGG TTC CGG ACA GGC TTG 7223  
 Arg Ala Leu Ser Asp Glu Val Ser Lys Trp Phe Arg Thr Gly Leu  
 2365 2370 2375

GGG GCC GAA CTG GAG GTG GCA CTA ACA TCT AGG TAT GAG GTA GAG 7268  
 Gly Ala Glu Leu Glu Val Ala Leu Thr Ser Arg Tyr Glu Val Glu  
 2380 2385 2390

GGC TGC AAA AGT ATC CTC ATA GCC ATG ACC ACC TTG GCG AGG GAC 7313  
 Gly Cys Lys Ser Ile Leu Ile Ala Met Thr Thr Leu Ala Arg Asp  
 2395 2400 2405

ATT AAG GCG TTT AAG AAA TTG AGA GGA CCT GTT ATA CAC CTC TAC 7358  
 Ile Lys Ala Phe Lys Lys Leu Arg Gly Pro Val Ile His Leu Tyr  
 2410 2415 2420

GGC GGT CCT AGA TTG GTG CGT TAA TACACAGAAT TCTGATTATA GCGCACTATT 7412  
 Gly Gly Pro Arg Leu Val Arg  
 2425 2430

ATAGCACC ATG AAT TAC ATC CCT ACG CAA ACG TTT TAC GGC CGC CGG 7459  
 Met Asn Tyr Ile Pro Thr Gln Thr Phe Tyr Gly Arg Arg  
 5 10

TGG CGC CCG CGC CCG GCG GCC CGT CCT TGG CCG TTG CAG GCC ACT 7504  
 Trp Arg Pro Arg Pro Ala Ala Arg Pro Trp Pro Leu Gln Ala Thr  
 15 20 25

CCG GTG GCT CCC GTC GTC CCC GAC TTC CAG GCC CAG CAG ATG CAG 7549  
 Pro Val Ala Pro Val Val Pro Asp Phe Gln Ala Gln Gln Met Gln  
 30 35 40

CAA CTC ATC AGC GCC GTA AAT GCG CTG ACA ATG AGA CAG AAC GCA 7594  
 Gln Leu Ile Ser Ala Val Asn Ala Leu Thr Met Arg Gln Asn Ala  
 45 50 55

SUBSTITUTE SHEET

Figure 5 (13)

17/33

```

ATT GCT CCT GCT AGG CCT CCC AAA CCA AAG AAG AAG AAG ACA ACC 7639
Ile Ala Pro Ala Arg Pro Pro Lys Pro Lys Lys Lys Lys Thr Thr
    60                65                70

AAA CCA AAG CCG AAA ACG CAG CCC AAG AAG ATC AAC GGA AAA ACG 7684
Lys Pro Lys Pro Lys Thr Gln Pro Lys Lys Ile Asn Gly Lys Thr
    75                80                85

CAG CAG CAA AAG AAG AAA GAC AAG CAA GCC GAC AAG AAG AAG AAG 7729
Gln Gln Gln Lys Lys Lys Asp Lys Gln Ala Asp Lys Lys Lys Lys
    90                95                100

AAA CCC GGA AAA AGA GAA AGA ATG TGC ATG AAG ATT GAA AAT GAC 7774
Lys Pro Gly Lys Arg Glu Arg Met Cys Met Lys Ile Glu Asn Asp
   105                110                115

TGT ATC TTC GAA GTC AAA CAC GAA GGA AAG GTC ACT GGG TAC GCC 7819
Cys Ile Phe Glu Val Lys His Glu Gly Lys Val Thr Gly Tyr Ala
   120                125                130

TGC CTG GTG GGC GAC AAA GTC ATG AAA CCT GCC CAC GTG AAA GGA 7864
Cys Leu Val Gly Asp Lys Val Met Lys Pro Ala His Val Lys Gly
   135                140                145

GTC ATC GAC AAC GCG GAC CTG GCA AAG CTA GCT TTC AAG AAA TCG 7909
Val Ile Asp Asn Ala Asp Leu Ala Lys Leu Ala Phe Lys Lys Ser
   150                155                160

AGC AAG TAT GAC CTT GAG TGT GCC CAG ATA CCA GTT CAC ATG AGG 7954
Ser Lys Tyr Asp Leu Glu Cys Ala Gln Ile Pro Val His Met Arg
   165                170                175

TCG GAT GCC TCA AAG TAC ACG CAT GAG AAG CCC GAG GGA CAC TAT 7999
Ser Asp Ala Ser Lys Tyr Thr His Glu Lys Pro Glu Gly His Tyr
   180                185                190

AAC TGG CAC CAC GGG GCT GTT CAG TAC AGC GGA GGT AGG TTC ACT 8044
Asn Trp His His Gly Ala Val Gln Tyr Ser Gly Gly Arg Phe Thr
   195                200                205

ATA CCG ACA GGA GCG GGC AAA CCG GGA GAC AGT GGC CGG CCC ATC 8089
Ile Pro Thr Gly Ala Gly Lys Pro Gly Asp Ser Gly Arg Pro Ile
   210                215                220

TTT GAC AAC AAG GGG AGG GTA GTC GCT ATC GTC CTG GGC GGG GCC 8134
Phe Asp Asn Lys Gly Arg Val Val Ala Ile Val Leu Gly Gly Ala
   225                230                235

AAC GAG GGC TCA CGC ACA GCA CTG TCG GTG GTC ACC TGG AAC AAA 8179
Asn Glu Gly Ser Arg Thr Ala Leu Ser Val Val Thr Trp Asn Lys
   240                245                250

GAT ATG GTG ACT AGA GTG ACC CCC GAG GGG TCC GAA GAG TGG TCC 8224
Asp Met Val Thr Arg Val Thr Pro Glu Gly Ser Glu Glu Trp Ser
   255                260                265

```

SUBSTITUTE SHEET

Figure 5 (14)

18/33

GCC CCG CTG ATT ACT GCC ATG TGT GTC CTT GCC AAT GCT ACC TTC	8269
Ala Pro Leu Ile Thr Ala Met Cys Val Leu Ala Asn Ala Thr Phe	
270 275 280	
CCG TGC TTC CAG CCC CCG TGT GTA CCT TGC TGC TAT GAA AAC AAC	8314
Pro Cys Phe Gln Pro Pro Cys Val Pro Cys Cys Tyr Glu Asn Asn	
285 290 295	
GCA GAG GCC ACA CTA CCG ATG CTC GAG GAT AAC GTG GAT AGG CCA	8359
Ala Glu Ala Thr Leu Arg Met Leu Glu Asp Asn Val Asp Arg Pro	
300 305 310	
GGG TAC TAC GAC CTC CTT CAG GCA GCC TTG ACG TGC CGA AAC GGA	8404
Gly Tyr Tyr Asp Leu Leu Gln Ala Ala Leu Thr Cys Arg Asn Gly	
315 320 325	
ACA AGA CAC CGG CGC AGC GTG TCG CAA CAC TTC AAC GTG TAT AAG	8449
Thr Arg His Arg Arg Ser Val Ser Gln His Phe Asn Val Tyr Lys	
330 335 340	
GCT ACA CGC CCT TAC ATC GCG TAC TGC GCC GAC TGC GGA GCA GGG	8494
Ala Thr Arg Pro Tyr Ile Ala Tyr Cys Ala Asp Cys Gly Ala Gly	
345 350 355	
CAC TCG TGT CAT AGC CCC GTA GCA ATT GAA GCG GTC AGG TCC GAA	8539
His Ser Cys His Ser Pro Val Ala Ile Glu Ala Val Arg Ser Glu	
360 365 370	
GCT ACC GAC GGG ATG CTG AAG ATT CAG TTC TCG GCA CAA ATT GGC	8584
Ala Thr Asp Gly Met Leu Lys Ile Gln Phe Ser Ala Gln Ile Gly	
375 380 385	
ATA GAT AAG AGT GAC AAT CAT GAC TAC ACG AAG ATA AGG TAC GCA	8629
Ile Asp Lys Ser Asp Asn His Asp Tyr Thr Lys Ile Arg Tyr Ala	
390 395 400	
GAC GGG CAC GCC ATT GAG AAT GCC GTC CCG TCA TCT TTG AAG GTA	8674
Asp Gly His Ala Ile Glu Asn Ala Val Arg Ser Ser Leu Lys Val	
405 410 415	
GCC ACC TCC GGA GAC TGT TTC GTC CAT GGC ACA ATG GGA CAT TTC	8719
Ala Thr Ser Gly Asp Cys Phe Val His Gly Thr Met Gly His Phe	
420 425 430	
ATA CTG GCA AAG TGC CCA CCG GGT GAA TTC CTG CAG GTC TCG ATC	8764
Ile Leu Ala Lys Cys Pro Pro Gly Glu Phe Leu Gln Val Ser Ile	
435 440 445	
CAG GAC ACC AGA AAC GCG GTC CGT GCC TGC AGA ATA CAA TAT CAT	8809
Gln Asp Thr Arg Asn Ala Val Arg Ala Cys Arg Ile Gln Tyr His	
450 455 460	
CAT GAC CCT CAA CCG GTG GGT AGA GAA AAA TTT ACA ATT AGA CCA	8854
His Asp Pro Gln Pro Val Gly Arg Glu Lys Phe Thr Ile Arg Pro	
465 470 475	

SUBSTITUTE SHEET

Figure 5 (15)

19/33

CAC TAT GGA AAA GAG ATC CCT TGC ACC ACT TAT CAA CAG ACC ACA	8899
His Tyr Gly Lys Glu Ile Pro Cys Thr Thr Tyr Gln Gln Thr Thr	
480 485 490	
CGC AAG ACC GTG GAG GAA ATC GAC ATG CAT ATG CCG CCA GAT ACG	8944
Ala Lys Thr Val Glu Glu Ile Asp Met His Met Pro Pro Asp Thr	
495 500 505	
CCG GAC AGG ACG TTG CTA TCA CAG CAA TCT GGC AAT GTA AAG ATC	8989
Pro Asp Arg Thr Leu Leu Ser Gln Gln Ser Gly Asn Val Lys Ile	
510 515 520	
ACA GTC GGA GGA AAG AAG GTG AAA TAC AAC TGC ACC TGT GGA ACC	9034
Thr Val Gly Gly Lys Lys Val Lys Tyr Asn Cys Thr Cys Gly Thr	
525 530 535	
GGA AAC GTT GGC ACT ACT AAT TCG GAC ATG ACG ATC AAC ACG TGT	9079
Gly Asn Val Gly Thr Thr Asn Ser Asp Met Thr Ile Asn Thr Cys	
540 545 550	
CTA ATA GAG CAG TGC CAC GTC TCA GTG ACG GAC CAT AAG AAA TGG	9124
Leu Ile Glu Gln Cys His Val Ser Val Thr Asp His Lys Lys Trp	
555 560 565	
CAG TTC AAC TCA CCT TTC GTC CCG AGA GCC GAC GAA CCG GCT AGA	9169
Gln Phe Asn Ser Pro Phe Val Pro Arg Ala Asp Glu Pro Ala Arg	
570 575 580	
AAA GGC AAA GTC CAT ATC CCA TTC CCG TTG GAC AAC ATC ACA TGC	9214
Lys Gly Lys Val His Ile Pro Phe Pro Leu Asp Asn Ile Thr Cys	
585 590 595	
AGA GTT CCA ATG GCG CGC GAA CCA ACC GTC ATC CAC GGC AAA AGA	9259
Arg Val Pro Met Ala Arg Glu Pro Thr Val Ile His Gly Lys Arg	
600 605 610	
GAA GTG ACA CTG CAC CTT CAC CCA GAT CAT CCC ACG CTC TTT TCC	9304
Glu Val Thr Leu His Leu His Pro Asp His Pro Thr Leu Phe Ser	
615 620 625	
TAC CGC ACA CTG GGT GAG GAC CCG CAG TAT CAC GAG GAA TGG GTG	9349
Tyr Arg Thr Leu Gly Glu Asp Pro Gln Tyr His Glu Glu Trp Val	
630 635 640	
ACA GCG GCG GTG GAA CGG ACC ATA CCC GTA CCA GTG GAC GGG ATG	9394
Thr Ala Ala Val Glu Arg Thr Ile Pro Val Pro Val Asp Gly Met	
645 650 655	
GAG TAC CAC TGG GGA AAC AAC GAC CCA GTG AGG CTT TGG TCT CAA	9439
Glu Tyr His Trp Gly Asn Asn Asp Pro Val Arg Leu Trp Ser Gln	
660 665 670	
CTC ACC ACT GAA GGG AAA CCG CAC GGC TGG CCG CAT CAG ATC GTA	9484
Leu Thr Thr Glu Gly Lys Pro His Gly Trp Pro His Gln Ile Val	
675 680 685	

SUBSTITUTE SHEET

Figure 5 (16)

20/33

CAG TAC TAC TAT GGG CTT TAC CCG GCC GCT ACA GTA TCC GCG GTC	9529
Gln Tyr Tyr Tyr Gly Leu Tyr Pro Ala Ala Thr Val Ser Ala Val	
690 695 700	
GTC GGG ATG AGC TTA CTG GCG TTG ATA TCG ATC TTC GCG TCG TGC	9574
Val Gly Met Ser Leu Leu Ala Leu Ile Ser Ile Phe Ala Ser Cys	
705 710 715	
TAC ATG CTG GTT GCG GCC CGC AGT AAG TGC TTG ACC CCT TAT GCT	9619
Tyr Met Leu Val Ala Ala Arg Ser Lys Cys Leu Thr Pro Tyr Ala	
720 725 730	
TTA ACA CCA GGA GCT GCA GTT CCG TGG ACG CTG GGG ATA CTC TGC	9664
Leu Thr Pro Gly Ala Ala Val Pro Trp Thr Leu Gly Ile Leu Cys	
735 740 745	
TGC GCC CCG CGG GCG CAC GCA GCT AGT GTG GCA GAG ACT ATG GCC	9709
Cys Ala Pro Arg Ala His Ala Ala Ser Val Ala Glu Thr Met Ala	
750 755 760	
TAC TTG TGG GAC CAA AAC CAA GCG TTG TTC TGG TTG GAG TTT GCG	9754
Tyr Leu Trp Asp Gln Asn Gln Ala Leu Phe Trp Leu Glu Phe Ala	
765 770 775	
GCC CCT GTT GCC TGC ATC CTC ATC ATC ACG TAT TGC CTC AGA AAC	9799
Ala Pro Val Ala Cys Ile Leu Ile Ile Thr Tyr Cys Leu Arg Asn	
780 785 790	
GTG CTG TGT TGC TGT AAG AGC CTT TCT TTT TTA GTG CTA CTG AGC	9844
Val Leu Cys Cys Cys Lys Ser Leu Ser Phe Leu Val Leu Leu Ser	
795 800 805	
CTC GGG GCA ACC GCC AGA GCT TAC GAA CAT TCG ACA GTA ATG CCG	9889
Leu Gly Ala Thr Ala Arg Ala Tyr Glu His Ser Thr Val Met Pro	
810 815 820	
AAC GTG GTG GGG TTC CCG TAT AAG GCT CAC ATT GAA AGG CCA GGA	9934
Asn Val Val Gly Phe Pro Tyr Lys Ala His Ile Glu Arg Pro Gly	
825 830 835	
TAT AGC CCC CTC ACT TTG CAG ATG CAG GTT GTT GAA ACC AGC CTC	9979
Tyr Ser Pro Leu Thr Leu Gln Met Gln Val Val Glu Thr Ser Leu	
840 845 850	
GAA CCA ACC CTT AAT TTG GAA TAC ATA ACC TGT GAG TAC AAG ACG	10024
Glu Pro Thr Leu Asn Leu Glu Tyr Ile Thr Cys Glu Tyr Lys Thr	
855 860 865	
GTC GTC CCG TCG CCG TAC GTG AAG TGC TGC GGC GCC TCA GAG TGC	10069
Val Val Pro Ser Pro Tyr Val Lys Cys Cys Gly Ala Ser Glu Cys	
870 875 880	
TCC ACT AAA GAG AAG CCT GAC TAC CAA TGC AAG GTT TAC ACA GGC	10114
Ser Thr Lys Glu Lys Pro Asp Tyr Gln Cys Lys Val Tyr Thr Gly	
885 890 895	

SUBSTITUTE SHEET

Figure 5 (17)

21/33

GTG TAC CCG TTC ATG TGG GGA GGG GCA TAT TGC TTC TGC GAC TCA	10159
Val Tyr Pro Phe Met Trp Gly Gly Ala Tyr Cys Phe Cys Asp Ser	
900 905 910	
GAA AAC ACG CAA CTC AGC GAG GCG TAC GTC GAT CGA TCG GAC GTA	10204
Glu Asn Thr Gln Leu Ser Glu Ala Tyr Val Asp Arg Ser Asp Val	
915 920 925	
TGC AGG CAT GAT CAC GCA TCT GCT TAC AAA GCC CAT ACA GCA TCG	10249
Cys Arg His Asp His Ala Ser Ala Tyr Lys Ala His Thr Ala Ser	
930 935 940	
CTG AAG GCC AAA GTG AGG GTT ATG TAC GGC AAC GTA AAC CAG ACT	10294
Leu Lys Ala Lys Val Arg Val Met Tyr Gly Asn Val Asn Gln Thr	
945 950 955	
GTG GAT GTT TAC GTG AAC GGA GAC CAT GCC GTC ACG ATA GGG GGT	10339
Val Asp Val Tyr Val Asn Gly Asp His Ala Val Thr Ile Gly Gly	
960 965 970	
ACT CAG TTC ATA TTC GGG CCG CTG TCA TCG GCC TGG ACC CCG TTC	10384
Thr Gln Phe Ile Phe Gly Pro Leu Ser Ser Ala Trp Thr Pro Phe	
975 980 985	
GAC AAC AAG ATA GTC GTG TAC AAA GAC GAA GTG TTC AAT CAG GAC	10429
Asp Asn Lys Ile Val Val Tyr Lys Asp Glu Val Phe Asn Gln Asp	
990 995 1000	
TTC CCG CCG TAC GGA TCT GGG CAA CCA GGG CGC TTC GGC GAC ATC	10474
Phe Pro Pro Tyr Gly Ser Gly Gln Pro Gly Arg Phe Gly Asp Ile	
1005 1010 1015	
CAA AGC AGA ACA GTG GAG AGT AAC GAC CTG TAC GCG AAC ACG GCA	10519
Gln Ser Arg Thr Val Glu Ser Asn Asp Leu Tyr Ala Asn Thr Ala	
1020 1025 1030	
CTG AAG CTG GCA CGC CCT TCA CCC GGC ATG GTC CAT GTA CCG TAC	10564
Leu Lys Leu Ala Arg Pro Ser Pro Gly Met Val His Val Pro Tyr	
1035 1040 1045	
ACA CAG ACA CCT TCA GGG TTC AAA TAT TGG CTA AAG GAA AAA GGG	10609
Thr Gln Thr Pro Ser Gly Phe Lys Tyr Trp Leu Lys Glu Lys Gly	
1050 1055 1060	
ACA GCC CTA AAT ACG AAG GCT CCT TTT GGC TGC CAA ATC AAA ACG	10654
Thr Ala Leu Asn Thr Lys Ala Pro Phe Gly Cys Gln Ile Lys Thr	
1065 1070 1075	
AAC CCT GTC AGG GCC ATG AAC TGC GCC GTG GGA AAC ATC CCT GTC	10699
Asn Pro Val Arg Ala Met Asn Cys Ala Val Gly Asn Ile Pro Val	
1080 1085 1090	
TCC ATG AAT TTG CCT GAC AGC GCC TTT ACC CGC ATT GTC GAG GCG	10744
Ser Met Asn Leu Pro Asp Ser Ala Phe Thr Arg Ile Val Glu Ala	
1095 1100 1105	

SUBSTITUTE SHEET



Figure 5 (18)

22/33

CCG ACC ATC ATT GAC CTG ACT TGC ACA GTG GCT ACC TGT ACG CAC 10789  
 Pro Thr Ile Ile Asp Leu Thr Cys Thr Val Ala Thr Cys Thr His  
 1110 1115 1120

TCC TCG GAT TTC GGC GGC GTC TTG ACA CTG ACG TAC AAG ACC AAC 10834  
 Ser Ser Asp Phe Gly Gly Val Leu Thr Leu Thr Tyr Lys Thr Asn  
 1125 1130 1135

AAG AAC GGG GAC TGC TCT GTA CAC TCG CAC TCT AAC GTA GCT ACT 10879  
 Lys Asn Gly Asp Cys Ser Val His Ser His Ser Asn Val Ala Thr  
 1140 1145 1150

CTA CAG GAG GCC ACA GCA AAA GTG AAG ACA GCA GGT AAG GTG ACC 10924  
 Leu Gln Glu Ala Thr Ala Lys Val Lys Thr Ala Gly Lys Val Thr  
 1155 1160 1165

TTA CAC TTC TCC ACG GCA AGC GCA TCA CCT TCT TTT GTG GTG TCG 10969  
 Leu His Phe Ser Thr Ala Ser Ala Ser Pro Ser Phe Val Val Ser  
 1170 1175 1180

CTA TGC AGT GCT AGG GCC ACC TGT TCA GCG TCG TGT GAG CCC CCG 11014  
 Leu Cys Ser Ala Arg Ala Thr Cys Ser Ala Ser Cys Glu Pro Pro  
 1185 1190 1195

AAA GAC CAC ATA GTC CCA TAT GCG GCT AGC CAC AGT AAC GTA GTG 11059  
 Lys Asp His Ile Val Pro Tyr Ala Ala Ser His Ser Asn Val Val  
 1200 1205 1210

TTT CCA GAC ATG TCG GGC ACC GCA CTA TCA TGG GTG CAG AAA ATC 11104  
 Phe Pro Asp Met Ser Gly Thr Ala Leu Ser Trp Val Gln Lys Ile  
 1215 1220 1225

TCG GGT GGT CTG GGG GCC TTC GCA ATC GGC GCT ATC CTG GTG CTG 11149  
 Ser Gly Gly Leu Gly Ala Phe Ala Ile Gly Ala Ile Leu Val Leu  
 1230 1235 1240

GTT GTG GTC ACT TGC ATT GGG CTC CGC AGA TAA GTTAGGGTAG 11192  
 Val Val Val Thr Cys Ile Gly Leu Arg Arg  
 1245 1250

GCAATGGCAT TGATATAGCA AGAAAATTGA AAACAGAAAA AGTTAGGGTA AGCAATGGCA 11252

TATAACCATA ACTGTATAAC TTGTAACAAA GCGCAACAAG ACCTGCGCAA TTGGCCCCGT 11312

GGTCGCCCTC ACGGAAACTC GGGGCAACTC ATATTGACAC ATTAATTGGC AATAATTGGA 11372

AGCTTACATA AGCTTAATTC GACGAATAAT TGGATTTTTA TTTTATTTTG CAATTGGTTT 11432

TTAATATTTT CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 11492

AAAAAAAAAA AAAAAAAAAA ACTAG 11517

SUBSTITUTE SHEET

23/33

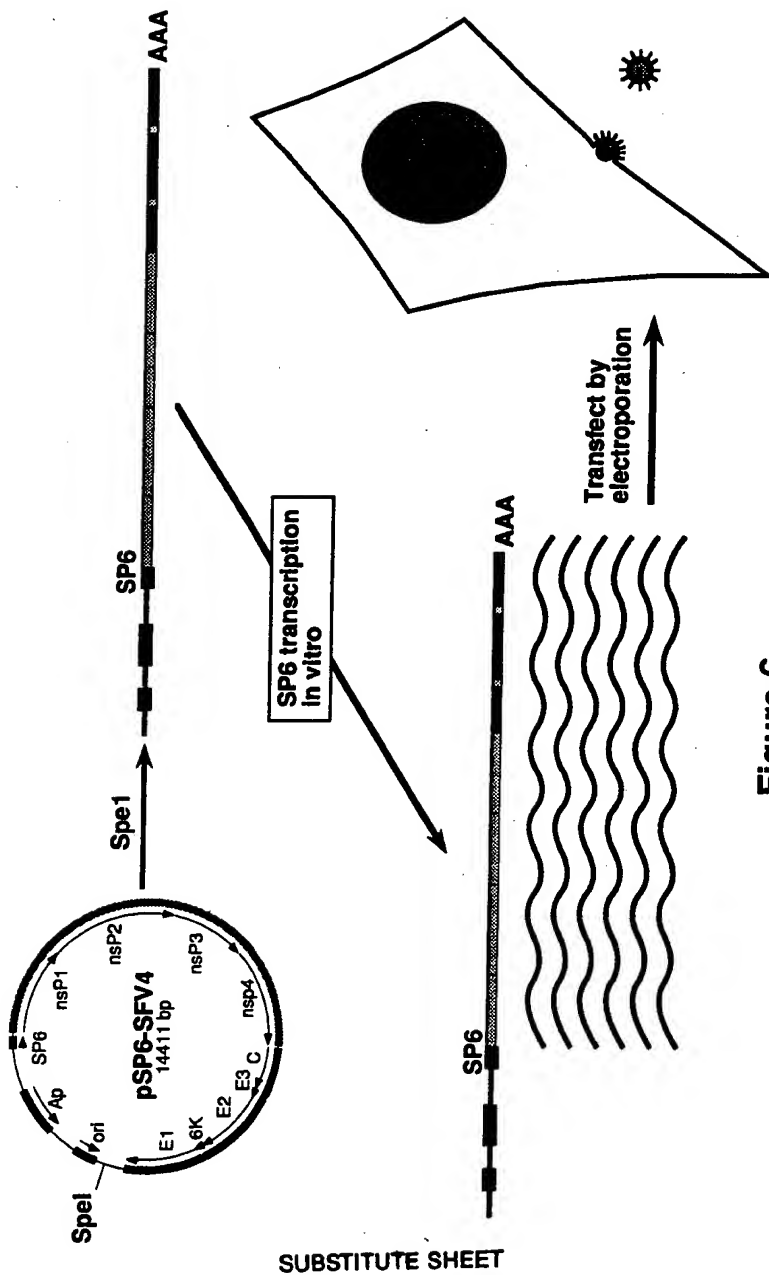


Figure 6

SUBSTITUTE SHEET

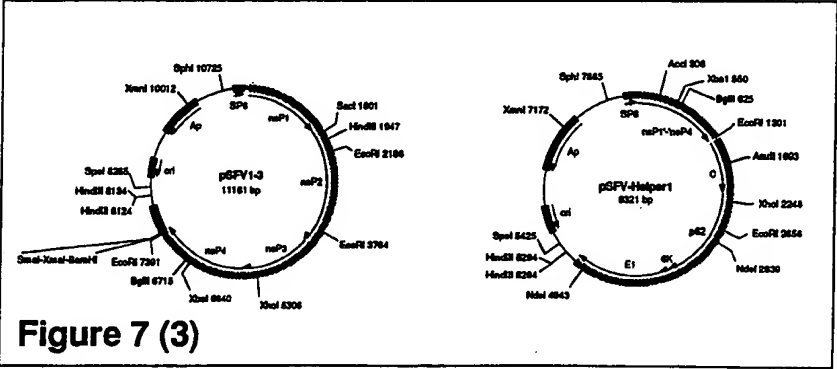
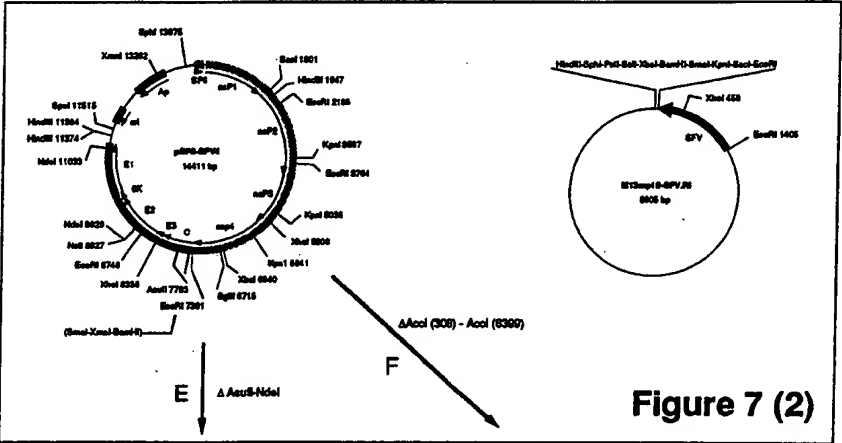
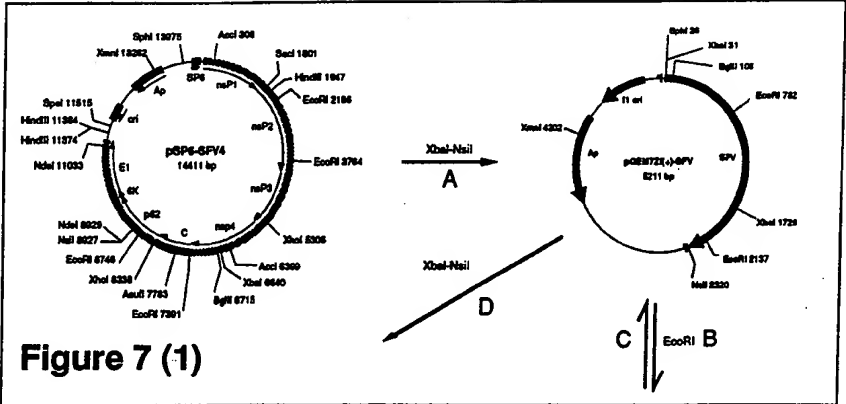


Figure 7 layout scheme

25/33

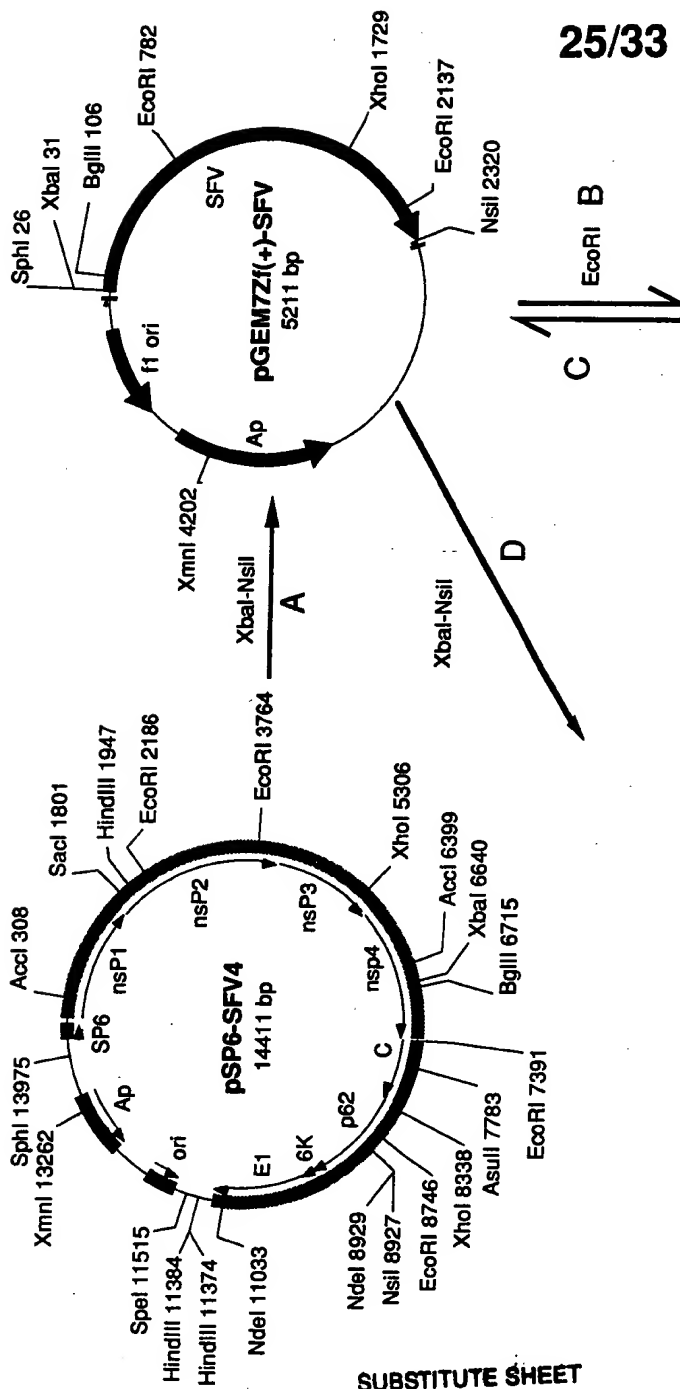


Figure 7 (1)

SUBSTITUTE SHEET

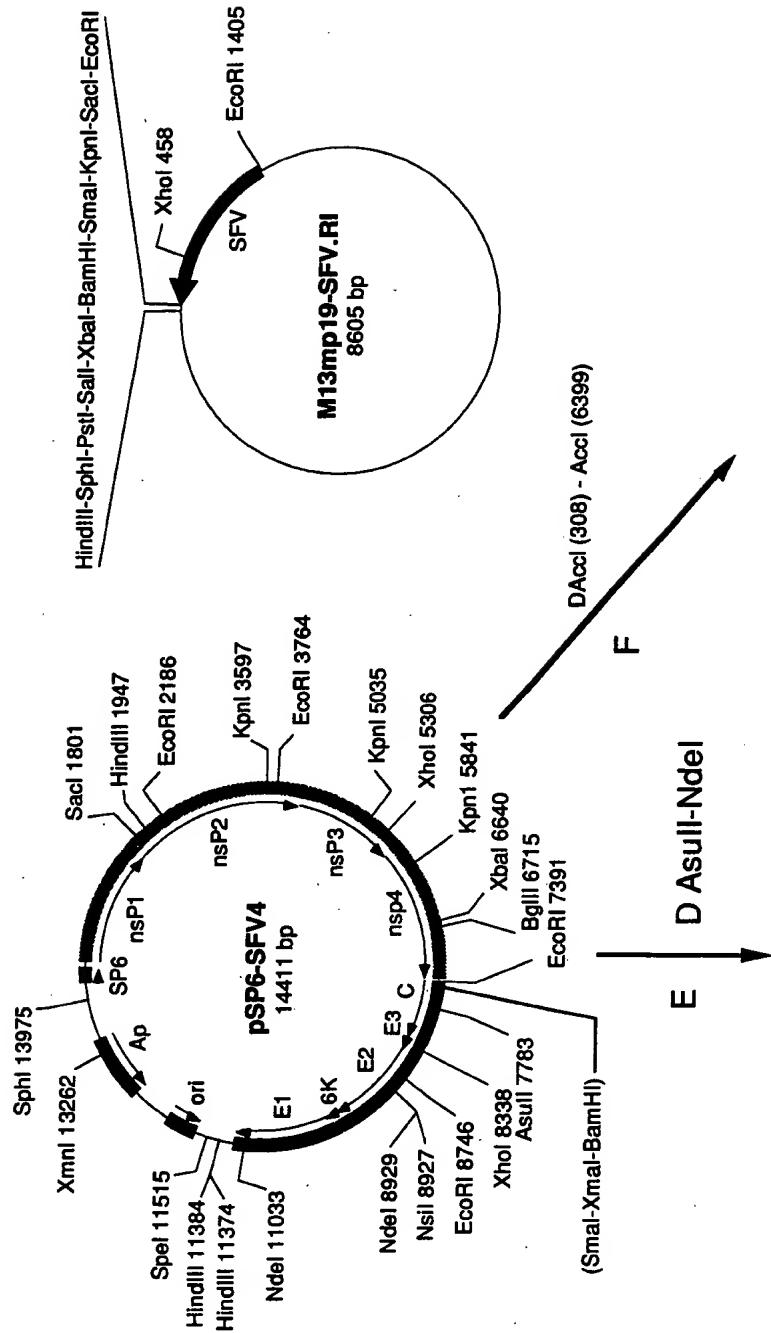


Figure 7 (2)

27/33

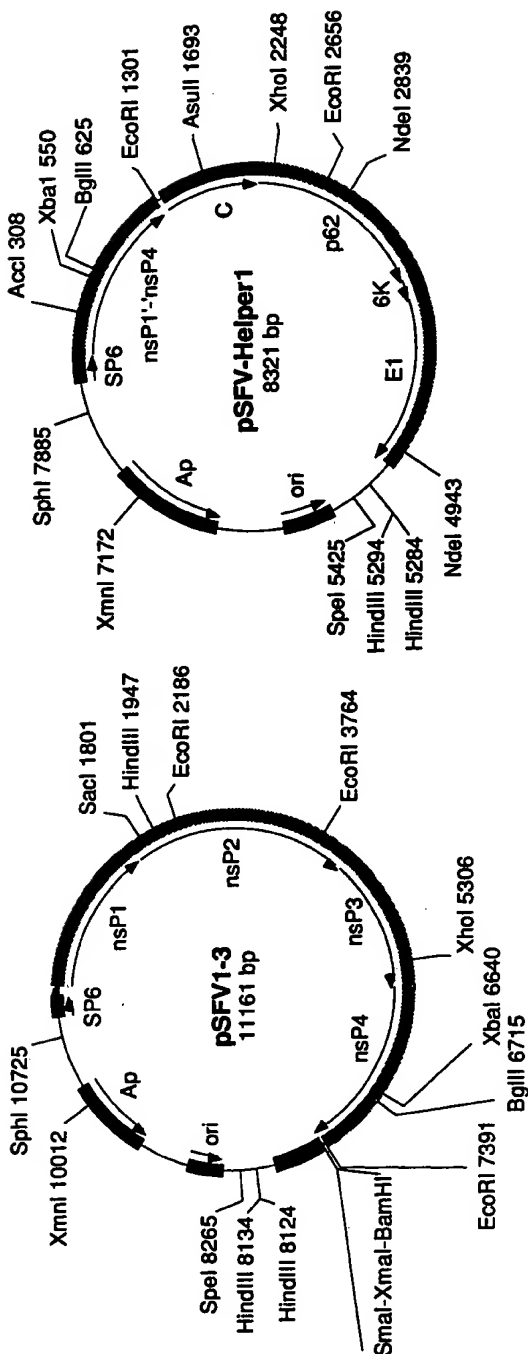


Figure 7 (3)

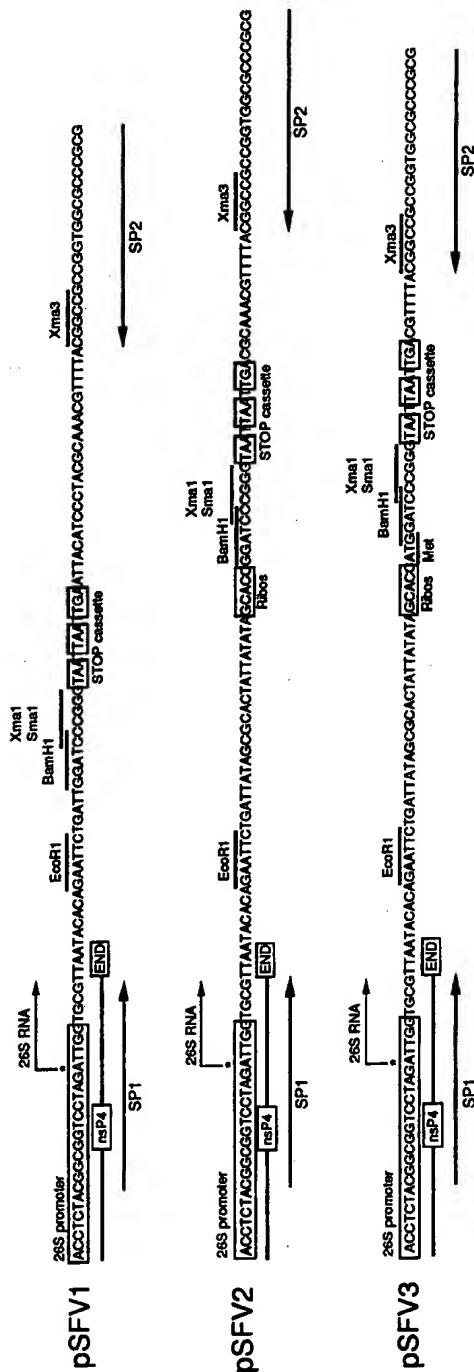


Figure 8

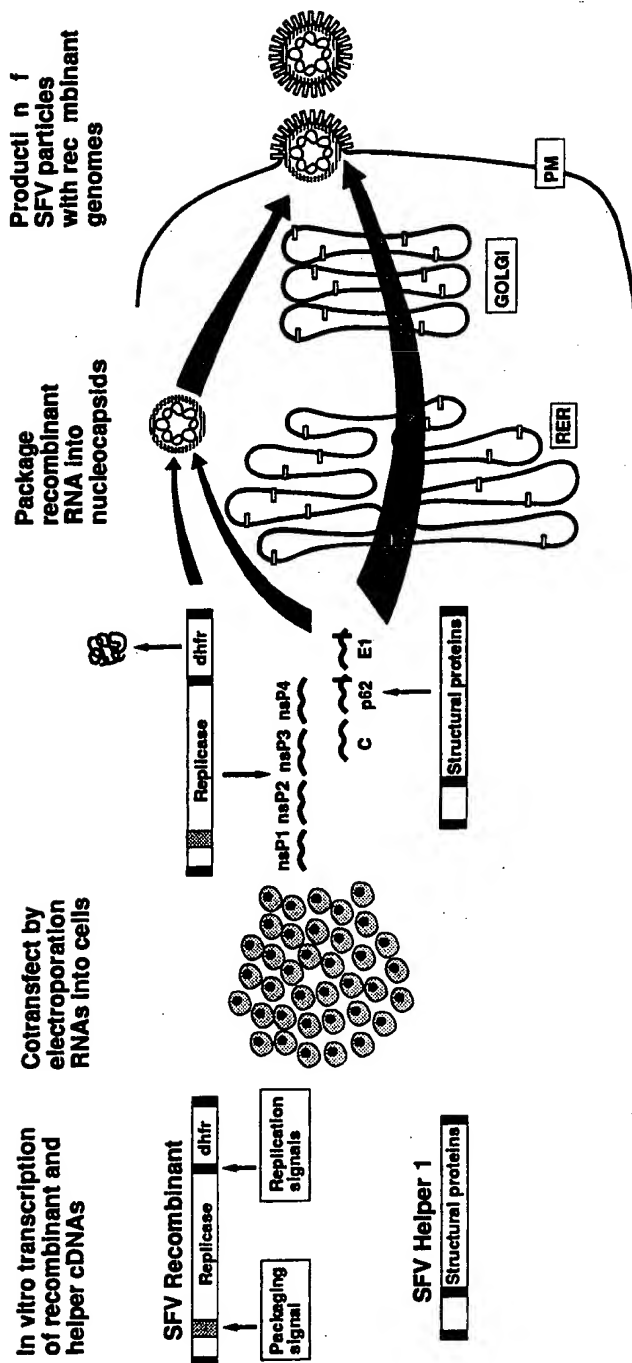


Figure 9



30/33

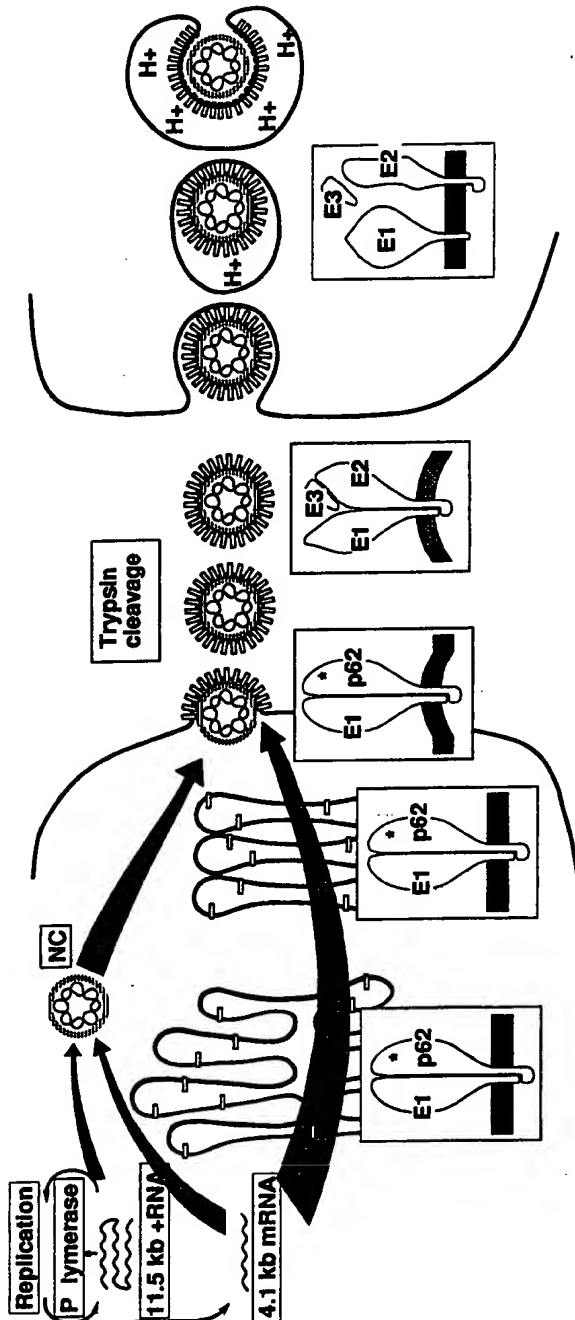


Figure 10

SUBSTITUTE SHEET

31/33

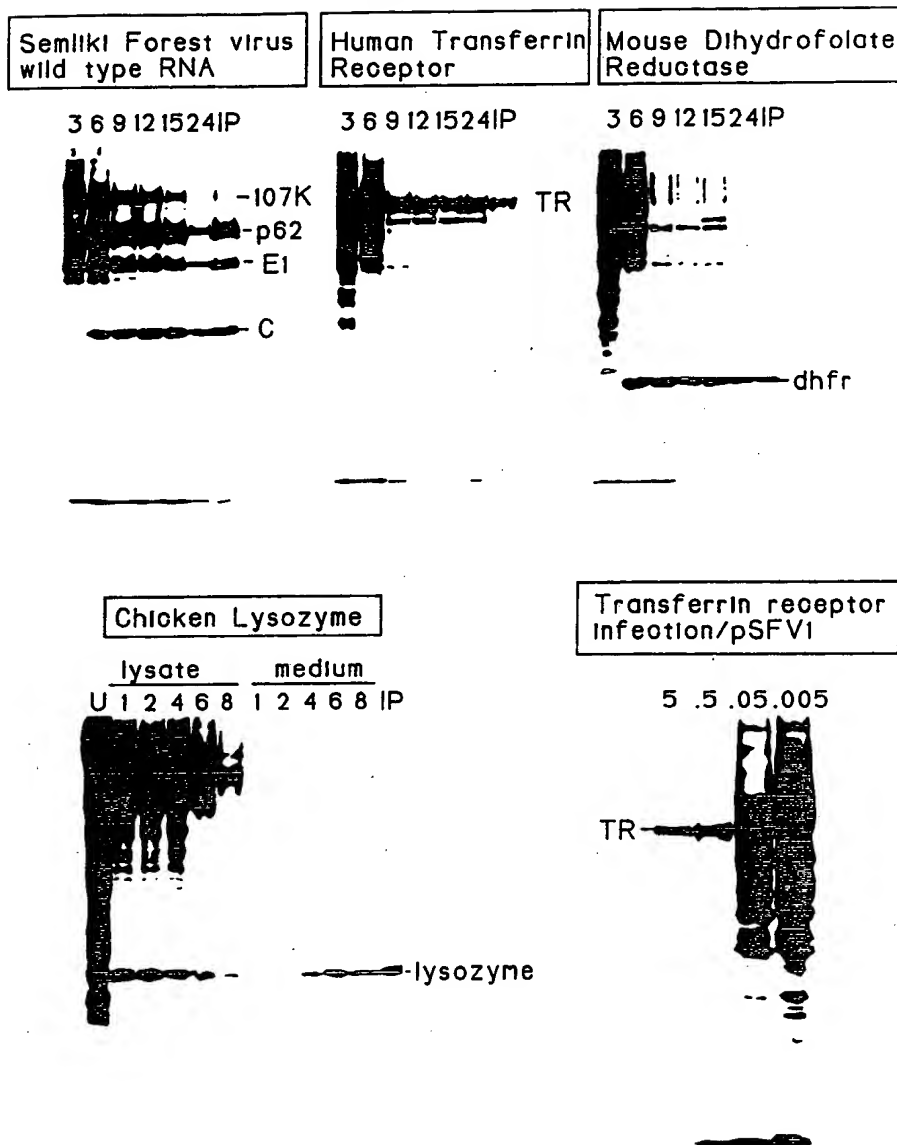


Figure 11

SUBSTITUTE SHEET

[illegible]

### Figure 12 (1)

33/33

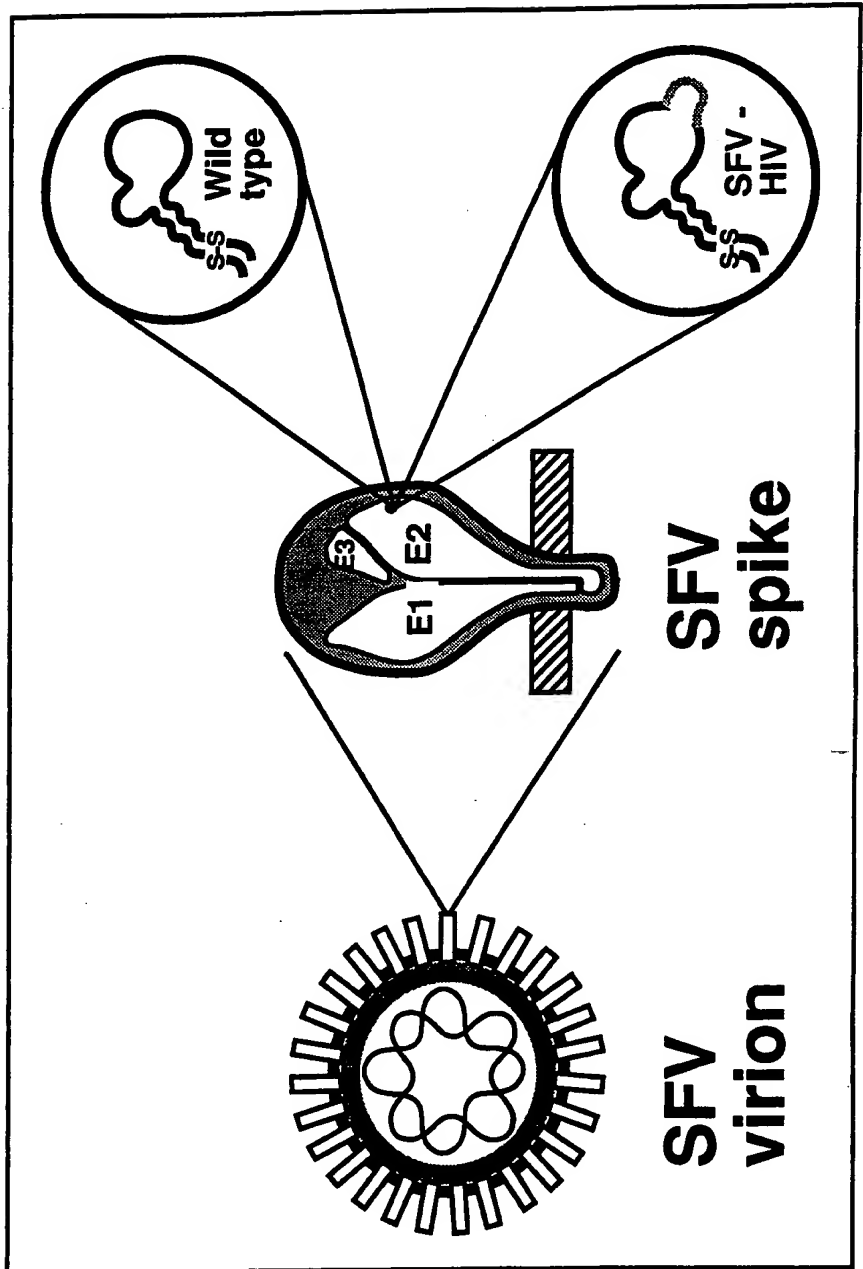


Figure 12 (2)

# INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00855

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>8</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC5: C 12 N 15/86, C 12 N 7/01, A 61 K 39/12</b>																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: right; padding-right: 20px;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Classification System</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Classification Symbols</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">IPC5</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">C 12 N; A 61 K</div> </td> </tr> </table>			<div style="border: 1px solid black; padding: 2px;">Classification System</div>	<div style="border: 1px solid black; padding: 2px;">Classification Symbols</div>	<div style="border: 1px solid black; padding: 2px;">IPC5</div>	<div style="border: 1px solid black; padding: 2px;">C 12 N; A 61 K</div>											
<div style="border: 1px solid black; padding: 2px;">Classification System</div>	<div style="border: 1px solid black; padding: 2px;">Classification Symbols</div>																
<div style="border: 1px solid black; padding: 2px;">IPC5</div>	<div style="border: 1px solid black; padding: 2px;">C 12 N; A 61 K</div>																
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched <sup>9</sup>  <b>SE,DK,FI,NO classes as above</b>																	
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category *</th> <th style="width: 70%; padding: 2px;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 20%; padding: 2px;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">PROC. NATL. ACAD. SCI., Vol. 84, 1987 (USA) Robin Levis et al: "Engineered defective interfering RNAs of Sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells", see page 4811 - page 4815 especially page 4811, column 1 lines 1-4,12-15,46-48; column 2 lines 25-28 and page 4812 column 1 lines 24-26 column 2 lines 14-16</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-2,5,9-11,20-22,29,30</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="text-align: center; vertical-align: top; padding: 5px;">3,4,6-8,12-19,23-28,31-41</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">WO, A1, 8912095 (APPLIED BIOTECHNOLOGY, INC.) 14 December 1989, see the whole document</td> <td style="text-align: center; vertical-align: top; padding: 5px;">3,4,6-8,12-19,23-28,31-41</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;"></td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td></td> </tr> </tbody> </table>			Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	PROC. NATL. ACAD. SCI., Vol. 84, 1987 (USA) Robin Levis et al: "Engineered defective interfering RNAs of Sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells", see page 4811 - page 4815 especially page 4811, column 1 lines 1-4,12-15,46-48; column 2 lines 25-28 and page 4812 column 1 lines 24-26 column 2 lines 14-16	1-2,5,9-11,20-22,29,30	Y	--	3,4,6-8,12-19,23-28,31-41	Y	WO, A1, 8912095 (APPLIED BIOTECHNOLOGY, INC.) 14 December 1989, see the whole document	3,4,6-8,12-19,23-28,31-41		--	
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>															
X	PROC. NATL. ACAD. SCI., Vol. 84, 1987 (USA) Robin Levis et al: "Engineered defective interfering RNAs of Sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells", see page 4811 - page 4815 especially page 4811, column 1 lines 1-4,12-15,46-48; column 2 lines 25-28 and page 4812 column 1 lines 24-26 column 2 lines 14-16	1-2,5,9-11,20-22,29,30															
Y	--	3,4,6-8,12-19,23-28,31-41															
Y	WO, A1, 8912095 (APPLIED BIOTECHNOLOGY, INC.) 14 December 1989, see the whole document	3,4,6-8,12-19,23-28,31-41															
	--																
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>a</sup> Special categories of cited documents:<sup>10</sup></p> <p><sup>A</sup> document defining the general state of the art which is not considered to be of particular relevance</p> <p><sup>E</sup> earlier document but published on or after the international filing date</p> <p><sup>L</sup> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p><sup>O</sup> document referring to an oral disclosure, use, exhibition or other means</p> <p><sup>P</sup> document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p><sup>T</sup> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p><sup>X</sup> document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p><sup>Y</sup> document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p><sup>&amp;</sup> document member of the same patent family</p> </div> </div>																	
<b>IV. CERTIFICATION</b> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">17th March 1992</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">1992 -03- 19</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">International Searching Authority</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">Signature of Authorized Officer</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;">SWEDISH PATENT OFFICE</div> </td> <td style="border: none; vertical-align: top;"> <div style="border: 1px solid black; padding: 2px;"> <i>Carolina Palmcrantz</i>            Carolina Palmcrantz         </div> </td> </tr> </table>			<div style="border: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</div>	<div style="border: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</div>	<div style="border: 1px solid black; padding: 2px;">17th March 1992</div>	<div style="border: 1px solid black; padding: 2px;">1992 -03- 19</div>	<div style="border: 1px solid black; padding: 2px;">International Searching Authority</div>	<div style="border: 1px solid black; padding: 2px;">Signature of Authorized Officer</div>	<div style="border: 1px solid black; padding: 2px;">SWEDISH PATENT OFFICE</div>	<div style="border: 1px solid black; padding: 2px;"> <i>Carolina Palmcrantz</i>            Carolina Palmcrantz         </div>							
<div style="border: 1px solid black; padding: 2px;">Date of the Actual Completion of the International Search</div>	<div style="border: 1px solid black; padding: 2px;">Date of Mailing of this International Search Report</div>																
<div style="border: 1px solid black; padding: 2px;">17th March 1992</div>	<div style="border: 1px solid black; padding: 2px;">1992 -03- 19</div>																
<div style="border: 1px solid black; padding: 2px;">International Searching Authority</div>	<div style="border: 1px solid black; padding: 2px;">Signature of Authorized Officer</div>																
<div style="border: 1px solid black; padding: 2px;">SWEDISH PATENT OFFICE</div>	<div style="border: 1px solid black; padding: 2px;"> <i>Carolina Palmcrantz</i>            Carolina Palmcrantz         </div>																

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	<p>Dialog Information Services, file 154, MEDLINE 85-91/July, Dialog accession no. 06757425, Medline accession no. 89059425, "Alphaviruses a new vector expressing heterologous genes", Vopr Virusol Jul-Aug 1988, 33 (4) p 502-4</p> <p>--</p> <p>-----</p>	1-2

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00855

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the Swedish Patent Office EDP file on 28/02/92  
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 8912095	89-12-14	AU-D- 3775789	90-01-05